University of Alberta

Studies of the Molecular Mechanisms of Mitochondrial Diseases Using the *Caenorhabditis elegans* Model System

by

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requirements for the degree of Doctor of Philosophy

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for my wife Jessica

and in loving memory of my grandparents,
Mertyl and Sam Greenberg,
Ruth and Max Grad

Abstract

Mitochondrial dysfunction, with an estimated incidence of 1 in 10,000 live births, is among the most common genetically determined conditions. Missense mutations in the human *NDUFV1* gene, which encodes the 51-kDa active site subunit of the NADH-ubiquinone oxidoreductase or complex I, can lead to severe neurological disorders. Owing to the complex nature of mitochondrial disorders, the mechanisms of pathogenesis of most mutations remain poorly understood. We have generated transgenic strains of *Caenorhabditis elegans* that express disease-causing mutations in the *nuo-1* gene, the *C. elegans* homologue of the *NDUFV1* gene. These strains demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent mitochondrial respiration. Surprisingly, cytochrome *c* oxidase activity and protein levels were reduced, establishing a connection between complexes I and IV.

Further examination of our *nuo-1* mutants revealed variable reductions in the steady-state levels of complex I subunits and a significant reduction in the amount of COXI subunit of complex IV. Assembly intermediates are observed for both complexes I and IV. Riboflavin supplementation promotes the assembly of both complexes I and IV and results in increased catalytic activities.

To redress the condition of lactic acidosis, we hypothesized that providing an alternate pathway for lactate oxidation would be beneficial in the treatment of mitochondrial dysfunction. The yeast gene *CYB2* encodes an L-lactate-cytochrome *c* oxidoreductase that oxidizes lactate to pyruvate and directly reduces cytochrome *c* without involving nicotinamide adenine dinucleotide (NAD) cofactors. We demonstrate that the heterologous expression of this enzyme in complex I-deficient strains of *C.*

elegans results in significant improvements in animal fitness. Live animal respiration rates and ATP contents are also substantially increased.

Our results indicate that complex I mutations exert their pathogenic effects in multiple ways: by impeding NADH metabolism, elevating lactate concentrations, interfering with the function and assembly of other mitochondrial respiratory chain complexes, and creating an energy deficit. Promoting complex I assembly with riboflavin results in the added benefit of partially reversing the complex IV deficit. Furthermore, providing an alternate pathway for lactate oxidation, via a gene therapy strategy, can produce significant benefits in complex I-associated disease.

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List of Abbreviations

A, Ala	alanine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ALS	amyotrophic lateral sclerosis
ANT	adenine nucleotide translocase
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
BN-PAGE	blue native polyacrylamide gel electrophoresis
BSA	bovine serum albumin
C	cysteine
COX	cytochrome *c* oxidase
DCA	dichloroacetate
DNA	deoxyribonucleic acid
E	glutamate
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
F_1	factor 1 of ATP synthase
FAD	flavin adenine dinucleotide
Fe-S	iron-sulfur cluster
FP	flavoprotein fraction of complex I
FMN	flavin mononucleotide
F_o	factor oligomycin of ATP synthase
G-3-P	glycerol-3-phosphate
GFP	green fluorescent protein
Glu	glutamate
Gly	glycine
HP	hydrophobic protein fraction of complex I
IP	iron-sulfur protein fraction of complex I
K	lysine
kb	kilobase
KCN	potassium cyanide
kDa	kilo Daltons
KSS	Kearns-Sayre syndrome
Leu	leucine
LHON	Leber hereditary optic neuroretinopathy
Lys	lysine
M	methionine, molar
MELAS	mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy with ragged red fibres
mdm	mitochondrial distribution and morphology mutant
mg	milligram
min	minutes

ml	milliliter
mM	millimolar
MnSOD	manganese superoxide dismutase
MRC	mitochondrial respiratory chain
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NAD$^+$	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP$^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NARP	neurogenic muscle weakness, ataxia, retinitis pigmentosa
nm	nanometer
NMR	nuclear magnetic resonance
OXPHOS	oxidative phosphorylation
P	proline
PCR	polymerase chain reaction
PDHC	pyruvate dehydrogenase complex
PEO	progressive external ophthalmoplegia
P_i	inorganic phosphate
Plet-858::*CYB2*	cytochrome *b*2 gene under the *let-858* promoter
Pnuo-1::*CYB2*	cytochrome *b*2 gene under the *nuo-1* promoter
Pro	proline
R	arginine
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal RNA
SAM	sorting and assembly machinery
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
Ser	serine
SOD	superoxide dismutase
T	threonine
TCA	trichloroacetic acid
TFAM	mitochondrial transcription factor A
TIM	translocase of the mitochondrial inner membrane
TOM	translocase of the mitochondrial outer membrane
tRNA	transfer RNA
Trp	tryptophan
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
V, Val	valine
X	premature stop codon
$\Delta\Psi$	mitochondrial inner membrane potential
μg	microgram
μl	microlitre

Chapter 1

General Introduction

1.1 Introduction

Mitochondrial diseases are often devastating disorders with complex presentations and multisystemic effects, making their diagnosis and treatment difficult. Compounding the severity of these diseases is the fact that there are no known cures. Although the last fifteen years have shown a dramatic increase in our understanding of mitochondrial dysfunction and its pathogenic effects, this has been paralleled by significant increases in the number of patients and families diagnosed with mitochondrial disorders. The demand for quicker and better understanding of these diseases has never been higher, challenging the creativity and intellect of mitochondrial researchers from around the world. As a result, research into mitochondrial disorders has branched into areas that were, until recently, untapped. This includes use of the nematode, *Caenorhabditis elegans*, a relatively new model system for the study of mitochondrial dysfunction and disease manifestation. The contents of this thesis describe a comprehensive study in which *C. elegans* was used to model human mutations that cause mitochondrial disease in order to better understand the molecular mechanisms of pathogenesis. The aim of this chapter is to introduce the reader to mitochondria and the mitochondrial respiratory chain (MRC), with particular attention given to the structure, function and assembly of mitochondrial complex I – the primary focus of this study. The chapter will also include succinct introductions into the general biology of mitochondria, human mitochondrial disorders and the *C. elegans* model system and its role in the study of MRC dysfunction.

1.2 Mitochondria

1.2.1 General Characteristics

Mitochondria are essential organelles present in almost all eukaryotes. Their primary function is to provide energy for the cell via the generation of adenosine triphosphate (ATP), the energy currency of the cell (Mitchell, 1987). Mitochondria are also the home of other important metabolic pathways such as the Krebs cycle, the urea cycle, the oxidation of fatty-acids, iron-sulfur cluster formation, and the biosynthesis of heme and lipids (Scheffler, 2000). First identified by light microscopy in the mid-nineteenth century as distinct cellular structures containing granules or filaments, mitochondria were originally believed to be autonomous living organisms that form bacteria-like colonies within the cytoplasm of host cells (Scheffler, 1999). Although this initial description was inaccurate, it carried with it some merit and fortuitous insight as it is now affirmed that mitochondria are direct descendants of a bacterial endosymobiont established inside a primitive eukaryote (Gray *et al.*, 1999). This symbiotic relationship resulted in the loss or transfer of genetic material between the bacterial genome and nucleus of the host eukaryote during evolution, resulting in the established dual genome state seen in eukaryotic cells today (Gray *et al.*, 2001).

Mitochondria are routinely represented in textbooks as a sausage-like organelle, resembling their typical appearance in human hepatocytes, with dimensions that average 3-4 μm in length and ~1 μm in diameter. At first glance, they resemble bacteria in size and shape (Scheffler, 1999). However, mitochondria can take on alternative forms, such as the continuous reticular network seen in the yeast *Saccharomyces cerevisiae* or the stacks of elongated tubules observed in *C. elegans* (Labrousse *et al.*, 1999). The number of mitochondria per cell varies widely from cell-type to cell-type; human hepatocytes routinely have 800 per cell, whereas lower eukaryotes, such as amoebas can have as many as 500,000 (Scheffler, 1999). For higher eukaryotes with differentiated cells, mitochondrial number can also vary depending on cell function or tissue-type; highly-energetic cells typically have greater numbers of mitochondria per cell than those with lower energy demands. Mitochondria have a common ultrastructure; a double membrane envelops the organelles: an outer membrane and an inner membrane (Figure 1.1). The mitochondrial inner membrane is extremely enriched in protein and is typically convoluted and folded into structures called cristae, which allow for a tremendous increase in the surface area of the membrane. In addition to the two membranes, which themselves constitute separate organellar environments, there is an intermembrane space sandwiched between the two membranes and an internal compartment called the matrix space. The mitochondrial outer membrane contains proteinaceous, porous structures called porins, which make the membrane permeable to small molecules of less than 10-kDa, such as ions and most metabolites (Benz, 1994; Jap and Walian, 1990). In contrast, the mitochondrial inner membrane is impermeable to most small molecules and ions, allowing only small, uncharged molecules to passively diffuse through it, making it the major barrier between the cytosol and the mitochondrial matrix (Ballarin *et al.*, 1996; Ballarin and Sorgato, 1996; Sorgato and Moran, 1993)

1.2.2 Morphology and Distribution

Mitochondria are dynamic structures that constantly undergo fission and fusion (Bereiter-Hahn and Voth, 1994). Mitochondrial fission is not unexpected since mitochondria are not synthesized *de novo*, but rather are derived from pre-existing organelles. Mitochondrial division and proliferation are essential parts of cell division, as they ensure that daughter cells inherit the new mitochondria necessary for viability (Yaffe, 1999). These processes also occur in non-dividing cells in response to increased cell volume, to environmental changes or to energy demands. The requirement for mitochondrial fusion is less obvious, although the mixing and exchange of proteins between fusing mitochondria have been seen experimentally (Nunnari *et al.*, 1997). Fusion is perhaps needed to acquire fresh membrane and protein when older organelles are damaged. Normal mitochondrial morphology is maintained as a result of an equilibrium between the processes of fission and fusion (Nunnari *et al.*, 1997). A collection of proteins has been identified that facilitate the process of fission, highlighted by the large transiently mitochondria-associated GTPase, Drp1 (Dnm1p in yeast) (Bleazard *et al.*, 1999; Otsuga *et al.*, 1998) and the mitochondrial outer membrane protein Fis1 (Jakobs *et al.*, 2003; Mozdy *et al.*, 2000). In mammalian cells, mitochondrial fusion is controlled primarily by the outer membrane GTPases Mfn1 and Mfn2 (Fzo1p in yeast) (Griparic and van der Bliek, 2001; Ishihara *et al.*, 2003; Mozdy and Shaw, 2003; Santel and Fuller, 2001) and the dynamin-related GTPase Opa1 (Mgm1p in yeast) (Olichon *et al.*, 2002; Satoh *et al.*, 2003; Sesaki *et al.*, 2003). Mutations in genes encoding any of these morphology factors can affect the equilibrium between fission and fusion resulting in either excessive mitochondrial connectivity (fission defects) or fragmentation (fusion defects).

In addition to their dynamic morphology, it is evident that mitochondria have a nonrandom distribution in cells rather than passively diffusing through the cytosol. It is now firmly established that mitochondrial shape and distribution are strongly influenced by their association with cytoskeletal components (Bereiter-Hahn and Voth, 1994; Rizzuto *et al.*, 1996). In higher eukaryotes, mitochondria can move rapidly along microtubules, likely via mitochondrion-specific motor proteins that control directional transport (Khodjakov *et al.*, 1998; Nangaku *et al.*, 1994; Pereira *et al.*, 1997). Mitochondria are also known to associate with intermediate filaments (Summerhayes *et al.*, 1983). In contrast, mitochondria from the budding yeast *S. cerevisiae* are tethered to the actin cytoskeleton (Fehrenbacher *et al.*, 2004). Much of what is known about the machinery responsible for mitochondrial segregation is derived from studies of the yeast *mdm* (mitochondrial distribution and morphology) mutants, which are defective in mitochondrial inheritance during cell division (Berger and Yaffe, 1996; Yaffe, 1999). A number of Mdm proteins are components of the mitochondrial outer membrane and are believed responsible for the interactions of mitochondria with the cytoskeleton, whereas others are found in the cytoplasm. One protein, Mdm1p even appears to be a novel component of the yeast cytoskeleton itself (McConnell and Yaffe, 1992; McConnell and Yaffe, 1993). The role of the cytoskeleton in regulating mitochondrial distribution during cell growth and differentiation and in response to metabolic demands remains poorly understood.

1.2.3. Biogenesis

New mitochondrial proteins must be continually synthesized, sorted and assembled into growing organelles. Estimates place the number of proteins that reside in the mitochondrion at 600 to 1,000 (Sickmann *et al.*, 2003; Taylor *et al.*, 2003). Although the mitochondrion carries its own genome and machinery for protein synthesis, only a few polypeptides, mostly constituents of the inner membrane, are actually synthesized in the matrix (Koehler, 2004). The remaining ~95% of mitochondrial proteins are synthesized on cytosolic ribosomes and imported into the mitochondrion. Mitochondrial proteins possess specific targeting and sorting information that directs them to their correct submitochondrial locations. Elaborate proteinaceous complexes are responsible for the recognition, translocation, sorting and assembly of incoming proteins. The TOM complex consists of receptors that bind and recognize incoming mitochondrial proteins and a translocation pore through which polypeptides can traverse the outer membrane to enter the intermembrane space (Koehler, 2004; Neupert, 1997; Pfanner *et al.*, 1997). The SAM complex facilitates the assembly of outer membrane proteins with complex topology, such as the β-barrel proteins porin and Tom40; the latter is the main component of the TOM translocation pore (Gentle *et al.*, 2004; Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003). Proteins imported to the intermembrane space via the TOM complex can subsequently take one of several routes. Soluble intermembrane space proteins may be met by a number of chaperones that mediate their proper folding and assembly (Davis *et al.*, 2000; Koehler, 2004; Sirrenberg *et al.*, 1998). Proteins destined for the matrix space are normally synthesized as precursor proteins with an N-terminal extension that is recognized by the TIM23 complex. TIM23 facilitates their translocation into the matrix space, where precursors are subsequently processed into their mature form, folded and assembled (Neupert, 1997; Pfanner *et al.*, 1997). Alternatively, some imported proteins that are destined to reside in the inner membrane are not synthesized with N-terminal extensions. They possess internal targeting information that is recognized by a second translocase, TIM22 (Curran *et al.*, 2002; Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). For the few polypeptides encoded in the mitochondrial genome and synthesized in the matrix, a number of components are needed to mediate their export and assembly into the inner membrane (He and Fox, 1999; Herrmann and Neupert, 2003; Souza *et al.*, 2000; Stuart, 2002). These include the integral inner membrane export factor Oxa1p (Bonnefoy *et al.*, 1994; Hell *et al.*, 1997), the peripheral assembly factor Mba1p (Rep and Grivell, 1996) and a group of proteins required specifically for the export and assembly of Cox2p (Broadley *et al.*, 2001; Souza *et al.*, 2000).

1.2.4 Mitochondrial DNA

Mammalian mitochondrial DNA (mtDNA) is a small, compact genome of circular double-stranded DNA that resides in the matrix space and is typically present in thousands of copies per cell. The human mitochondrial genome has a length of 16.6 kb, while the *C. elegans* mtDNA is just under 14 kb (Okimoto *et al.*, 1992). Mammalian mtDNA encodes two ribosomal RNAs (rRNA), 12S-rRNA and 16S-rRNA, 22 transfer RNAs (tRNAs) and 13 polypeptides of the mitochondrial respiratory chain (MRC): seven subunits of complex I (NADH-ubiquinone oxidoreductase), one subunit of complex III (ubiquinol-cytochrome *c* oxidoreductase), three subunits of complex IV (cytochrome *c* oxidase) and two subunits of complex V (ATP synthase) (Figure 1.2) (Attardi and Montoya, 1983; Attardi and Schatz, 1988). The *C. elegans* mtDNA differs in gene composition by having only one gene for complex V subunits (see section 1.3). Neither the nematode nor the human mtDNAs have introns but both have a region devoid of genes, referred to as the displacement- or D-loop, which is involved in mtDNA replication and transcription (Shadel and Clayton, 1997). Multiple copies of mtDNA are anchored to the mitochondrial inner membrane by a poorly defined set of proteins, forming structures called nucleoids (Albring *et al.*, 1977; Barat *et al.*, 1985; Newman *et al.*, 1996). It is believed that the nucleoids are the units of inheritance that must be properly distributed and segregated during mitochondrial division and proliferation (Jacobs *et al.*, 2000).

The replication of mtDNA is only loosely coupled to nuclear DNA replication. The precise mechanisms regulating mtDNA copy number have yet to be elucidated, although the process of mtDNA replication is relatively well understood, especially in mammalian mitochondria (Scheffler, 1999; Scheffler, 2000). Mammalian mtDNA has two origins of DNA replication, one for each strand. Due to the asymmetric distribution of guanosine plus cytosine nucleotides in mammalian mtDNA, the strands are denoted as either "light" (L) or "heavy" (H). The origin of H-strand synthesis (O_H) is located within the D-loop region, while the origin of L-strand synthesis (O_L) is located approximately half-way around the circular genome from the O_H. The strands are replicated independently, with the initiation and elongation of the H-strand occurring first. Replication is initiated at O_H by an RNA primer transcribed by a mitochondrial RNA polymerase from the L-strand promoter (LSP) located within the D-loop region (Larsson *et al.*, 1998; Shadel and Clayton, 1997). The primer is subsequently elongated by mitochondrial DNA polymerase γ. Separation of the H-strand from the L-strand by replication forms the D-loop, which can be clearly identified by microscopic analysis. As elongation of the H-stand proceeds, strand displacement will pass through O_L, allowing the initiation of L-strand synthesis with a second RNA primer. Replication is completed by removal of the RNA primers and ligation to form circular DNA molecules.

In the mammalian system, three transcription factors are essential for mtDNA transcription and maintenance, mitochondrial transcription factor A (TFAM) (Larsson *et al.*, 1998) and mitochondrial transcription factors B1 (TFB1M) and B2 (TFB2M) (Falkenberg *et al.*, 2002; Rantanen *et al.*, 2003). Transcription of mtDNA typically starts from two promoter regions, one for each strand, located close within the D-loop control region. Transcription from these promoter sites either generates short transcripts, which are used as RNA primers for DNA replication, or longer transcripts that carry long polycistronic messages. Termination of transcription is controlled by a mitochondrial transcription termination factor (Micol *et al.*, 1996). Endonucleolytic cleavage of the long primary transcripts produces the 22 tRNAs, 2 rRNAs and 13 mRNAs (Attardi and Montoya, 1983).

The most striking difference between mitochondrial and cytosolic translation is the use of an alternate genetic code by the mitochondrial translational machinery. The standard code recognizes the UGA codon as a stop codon but in metazoan and fungal mitochondria, it is translated as a Trp residue. The AGA codon, which specifies an Arg residue in the standard code, is read as a stop codon in mammalian mitochondria and as a Ser residue in the mitochondria of the fruit fly *Drosophila melanogaster*. In all, nine codons are known to specify alternative information in the mitochondria of various organisms (Scheffler, 1999).

Most ribosomal proteins and translation factors such as initiation and elongation factors are encoded in the nucleus and imported from the cytosol. Although functionally homologous, the components of the mitochondrial translational machinery differ considerably from their cytosolic counterparts. While some mitochondrial ribosomal proteins share significant sequence identity with their cytosolic counterparts, many are unique to the mitochondrial machinery (Cavdar Koc *et al.*, 2001; Graack and Wittmann-Liebold, 1998; O'Brien *et al.*, 1999; Suzuki *et al.*, 2001). It has yet to be shown that all components of a mitochondrial protein translation system have been identified in any organism.

1.3 Mitochondrial Respiratory Chain

1.3.1 Oxidative Phosphorylation

The primary function of mitochondria is to generate cellular energy via the process of oxidative phosphorylation (OXPHOS). OXPHOS requires the MRC, five multisubunit protein complexes embedded in the mitochondrial inner membrane. The MRC consists of ~80 structural subunits and approximately two dozen cofactors. It requires a number of chaperones and assembly factors for proper function and assembly (Hatefi, 1985; Mitchell, 1987). The biogenesis and regulation of the MRC is complex, involving both the nuclear and mitochondrial genomes. The mammalian and nematode MRCs consist of the following enzymes: the NADH-ubiquinone oxidoreductase (complex I), the succinate-ubiquinone oxidoreductase (succinate dehydrogenase; complex II), the ubiquinol-cytochrome *c* oxidoreductase (cytochrome bc_1 complex; complex III), the cytochrome *c* oxidase (complex IV), and the ATP synthase (F_0F_1-ATPase; complex V) (Figure 1.3). Although usually depicted as five discrete complexes within the mitochondrial inner membrane, there is increasing evidence suggesting direct physical interactions between MRC complexes resulting in 'supercomplexes' (Eubel *et al.*, 2004; Schägger *et al.*, 2004; Schägger and Pfeiffer, 2000). The structures of supercomplexes vary between organisms, but they are all believed to enhance catalytic efficiency, to channel substrates and to stabilize the individual complexes.

The MRC transports electrons derived from the oxidation of nutrients such as glucose and fatty acids along a series of cofactors of increasing reduction potential. The electrons are ultimately received by molecular oxygen, which is reduced to water. During electron transport by complexes I, III, and IV, protons are pumped across the inner membrane into the intermembrane space, storing energy as a proton gradient. The proton gradient is comprised of both a pH difference (ΔpH) and a charge difference or membrane potential ($\Delta\psi$). Complex V can utilize the energy of the proton gradient to catalyze ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate (P_i). ATP is transported from the matrix to the intermembrane space via the adenine nucleotide translocase (ANT) in exchange for ADP (Stubbs *et al.*, 1978; Vignais, 1976).

1.3.2 NADH-Ubiquinone Oxidoreductase (Complex I)

Complex I, the largest and most structurally intricate of the MRC enzymes, serves as the main entry point for electrons into the MRC (Friedrich *et al.*, 1998; Walker, 1992; Weiss *et al.*, 1991; Yagi *et al.*, 1998). The bovine heart enzyme is the best characterized; it is composed of at least 46 subunits, seven of which are encoded by the mtDNA, a non-covalently bound flavin mononucleotide (FMN) cofactor and at least eight iron-sulfur clusters. The bovine enzyme has an estimated mass of 980-kDa if a single copy of each subunit is assembled into the holoenzyme (Carroll *et al.*, 2003). In addition to bovine complex I, much study has been dedicated to the characterization of the enzyme from *Neurospora crassa* (Videira and Duarte, 2001; Videira and Duarte, 2002), *Yarrowia lipolytica* (Djafarzadeh *et al.*, 2000) and several plants (Combettes and Grienenberger, 1999; Rasmusson *et al.*, 1998), providing valuable insight into its functional and structural evolution.

Complex I oxidizes reduced nicotinamide adenine dinucleotide (NADH) to NAD^+, first transferring the two electrons to FMN and then along its series of iron-sulfur clusters and eventually to the lipid-soluble carrier ubiquinone (coenzyme Q). Reduced ubiquinone, or ubiquinol, shuttles electrons from complex I to complex III (Chazotte and

Hackenbrock, 1989; Zhu *et al.*, 1982), where it is re-oxidized to ubiquinone. Transfer of an electron pair through complex I is coupled to the translocation of four protons across the mitochondrial inner membrane (Brown and Brand, 1988).

A low-resolution structure of the *N. crassa* complex I was determined from two-dimensional crystals and non-crystalline detergent-solubilized preparations by electron microscopy (Hofhaus *et al.*, 1991; Leonard *et al.*, 1987). Subsequently, three-dimensional structures of the *N. crassa* and bovine enzymes were determined by electron cryomicroscopy (Grigorieff, 1998; Guenebaut *et al.*, 1997). Complex I has an L-shaped structure with one arm embedded in the mitochondrial inner membrane and the other protruding into the matrix space (Figure 1.4). The bovine structure, solved to 22 Å, currently provides the highest resolution information and differs slightly from the *N. crassa* structure in that the matrix-protruding arm is larger and more globular and has a thin stalk region linking it to the membrane arm.

The arrangement of the subunits within complex I cannot be discerned from the currently available low resolution structures. However, studies involving the fractionation or solubilization of the complex, along with some genetic investigations have provided clues to the approximate location of groups of subunits within the holoenzyme. Disruption of complex I by chaotropic anions, such as perchlorate generates three distinct fractions referred to as the flavoprotein (FP), iron-protein (IP) and hydrophobic protein (HP) fractions (Galante and Hatefi, 1979). The FP and IP fractions are generally assigned to the matrix arm of the enzyme, while the HP fraction logically constitutes the membrane-embedded arm. More recent studies have resolved complex I into three different subcomplexes that collectively contain most of the known subunits:

Iα constitutes the extrinsic arm and part of the membrane arm; Iβ constitutes a substantial portion of the membrane arm; Iλ is a derivative of the Iα subcomplex that only contains components of the extrinsic arm (Sazanov *et al.*, 2000). Subcomplex Iα retains the capacity for NADH oxidation and electron transfer to ubiquinone, indicating that it contains all of the subunits involved in substrate and cofactor binding. Fourteen subunits are believed to be essential for full complex I activity (Walker, 1992). These are referred to as 'core' subunits and consist of all seven mtDNA-encoded subunits (ND1-ND6, ND4L) and seven nuclear-encoded subunits (the 75-, 51-, 49-, 30-, 24-kDa, PSST, and TYKY subunits). All core subunits are highly conserved through evolution (Hirst *et al.*, 2003).

Five of the seven nuclear-encoded core subunits contain electron-transporting cofactors. The 51-kDa subunit, the focus of my studies in this thesis, is of particular functional importance as it contains a [4Fe-4S] cluster and the FMN- and NADH-binding sites (Chen and Guillory, 1981; Galante and Hatefi, 1979; Yano *et al.*, 1996). The 51- and 24-kDa subunits are believed to be in close proximity to one another and are both found in the FP subcomplex (Galante and Hatefi, 1979). The 75-kDa subunit, which carries three iron-sulfur centers, is likely closely associated with the 51- and 24-kDa subunits to facilitate efficient electron transport (Hirst *et al.*, 2003). The 49-kDa and PSST subunits are thought to also closely associate with each other because of their sequence homology to a pair of interacting components in NiFe hydrogenases (Albracht, 1993). The PSST subunit is believed to harbor the terminal iron-sulfur cluster that donates electrons to ubiquinone (Ahlers *et al.*, 2000; Kashani-Poor *et al.*, 2001; Magnitsky *et al.*, 2002; Ohnishi, 1998; Schuler *et al.*, 1999). Less is known about the relative positions of the TYKY and 30-kDa subunits. The TYKY subunit carries at least two iron-sulfur centers (Rasmussen *et al.*, 2001), while little is known of the function of the 30-kDa subunit (Hirst *et al.*, 2003). All the mtDNA-encoded subunits (ND1-ND6, ND4L) are integral membrane proteins containing transmembrane helices. The precise functions of the mtDNA-encoded subunits remain poorly understood but some appear to be involved in catalytic activity or in the assembly of complex I (Bai *et al.*, 2004; Chomyn, 2001). The remaining nuclear-encoded subunits are referred to as "supernumerary' subunits. Their sequences are less conserved than those of the core subunits and their functions remain unclear (Hirst *et al.*, 2003).

Interestingly, little is known of the mechanisms responsible for assembly of the complex I holoenzyme. The most comprehensive studies have been conducted in *N. crassa* (Schulte *et al.*, 1994). In this organism, complex I appears to be assembled in a step-wise fashion from a series of different modules or building blocks (Videira, 1998). A similar mechanism has been proposed for the human enzyme (Vogel *et al.*, 2004). To date, only two chaperone proteins involved in the assembly of complex I have been identified in *N. crassa.* CIA30 and CIA84 are tightly and specifically associated with membrane arm intermediates during the assembly process but are not part of the fully assembled complex (Küffner *et al.*, 1998). The CIA30 protein is moderately conserved through evolution, with homologues identified in organisms such as humans and *C. elegans* (Janssen *et al.*, 2002). Mitochondrial acyl carrier proteins also appear required for proper assembly of the peripheral and membrane arms of complex I in *N. crassa* (Schulte, 2001) but the details of this mechanism remain elusive.

1.3.3 Succinate-Ubiquinone Oxidoreductase (Complex II)

Complex II is structurally the simplest of all the MRC complexes. It provides a second site of entry for electrons into the respiratory chain, in addition to its role as a component of the Krebs cycle (Horsefield *et al.*, 2004; Lemire and Oyedotun, 2002). Complex II consists of four nuclear-encoded subunits, making it the only MRC complex to be entirely derived from one genome. The four subunits are assembled into a complex of ~130 kDa. The human nuclear genes *SDHA* and *SDHB* encode a catalytic heterodimer containing a covalently-bound flavin adenine dinucleotide (FAD) cofactor and three iron-sulfur clusters. The catalytic subunits are anchored to the matrix face of the mitochondrial inner membrane by two hydrophobic, heme-containing proteins encoded by the *SDHC* and *SDHD* genes. The anchor subunits also contain two ubiquinone binding sites.

Electrons from the oxidation of succinate to fumarate enter complex II through the FAD in the SDHA subunit. The iron-sulfur clusters form an almost linear chain from the FAD to the first quinone-binding site, where ubiquinone is reduced to ubiquinol. The roles of the heme and the second quinone-binding site are still enigmatic. Complex II does not directly contribute to the proton motive force, as it does not translocate protons during electron transfer.

1.3.4 Ubiquinol-Cytochrome c Oxidoreductase (Complex III)

Complex III couples proton pumping to the transfer of electrons from ubiquinol to cytochrome *c* (Trumpower, 1990; Trumpower and Gennis, 1994). The mammalian enzyme exists as a homodimer of ~240-kDa, with each monomer comprising 11 subunits (Schultz and Chan, 2001). The cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur subunits, which contain all of the electron-transferring cofactors of the enzyme, constitute the catalytic core of the complex (Saraste, 1999). Cytochrome *b*, the sole mtDNA-encoded subunit, contains two six-coordinate low-spin *b*-type hemes, b_L and b_H (low and high potential hemes, respectively) and two quinone-binding sites designated Q_i and Q_o for in and out, respectively. The Rieske iron-sulfur protein contains a high potential [2Fe-2S] cluster and cytochrome c_I contains a six-coordinate low-spin *c* heme that serves as the electron donor to cytochrome *c*, a soluble, mobile protein of the intermembrane space. The roles of other complex III subunits are not well understood but are required for assembly or stability of the enzyme (Scheffler, 1999).

Electron transfer within complex III is more complicated than the linear pathway in complexes II, involving the Q-cycle, a branched electron transfer pathway (Brandt and Trumpower, 1994). One electron is transferred from ubiquinol bound at the Q_o site along a high-potential path to the Rieske iron-sulfur center and cytochrome c_I. The second electron is transferred to the hemes and the Q_i site, where it is used to reduce ubiquinone to a ubisemiquinone or in a second round, ubisemiquinone to ubiquinol. The net effect is that two ubiquinol molecules are oxidized in each reaction cycle, reducing two molecules of cytochrome *c* and regenerating one molecule of ubiquinol.

1.3.5 Cytochrome c Oxidase (Complex IV)

Complex IV catalyzes the transfer of electrons from cytochrome *c* to oxygen, producing water (Malatesta *et al.*, 1995; Michel *et al.*, 1998; Musser *et al.*, 1995). Mammalian complex IV, with a molecular weight of ~200-kDa, is comprised of 13 subunits, three of which, COXI-III are encoded in the mitochondrial genome. COXI-III are highly conserved and carry all the redox-active metal centers involved in electron transfer. Subunit I contains a copper atom, Cu_B and two hemes, a six-coordinate low-spin heme *a* and a five-coordinate high-spin heme a_3. Heme a_3 and Cu_B interact, forming a binuclear site at which oxygen binds and is reduced (Babcock and Wikstrom, 1992; Capaldi, 1990). Subunit II contains a binuclear copper center, referred to as Cu_A, which serves as the initial electron acceptor from cytochrome *c* (Capaldi, 1990). Electrons from Cu_A are subsequently transferred to heme *a* and the a_3-Cu_B redox center. The function of subunit III remains unknown, possibly modulating proton pumping (Brunori *et al.*, 1987). The other 10 nuclear-encoded subunits are thought to modulate the assembly or catalytic properties of the holoenzyme (Poyton *et al.*, 1988). Seven of these subunits (IV, VIa, VIc, VIIa, VIIb, VIIc, and VIII) are small, integral membrane proteins; subunits Va, Vb, and VIb are membrane-extrinsic components (Tsukihara *et al.*, 1996). Subunit Vb contains a bound zinc ion, which likely serves a structural role in the protein. Complex IV utilizes four protons from the matrix to form water and pumps an additional four from the matrix to the intermembrane space. Two hydrophilic channels connect the active site to the aqueous environment on the matrix side and are believed to facilitate proton pumping (Wikström, 1998). Catalysis by complex IV results in the pumping of one proton into the intermembrane space for every electron transferred from cytochrome *c*.

1.3.6 ATP Synthase (Complex V)

Complex V utilizes the proton motive force to synthesize ATP from ADP and P_i. Structurally, it resembles a turbine; the flow of protons promotes rotation of a portion of the enzyme and ATP synthesis. The enzyme is comprised of two assemblies: a globular catalytic assembly known as F_1 that protrudes into the matrix and an integral membrane assembly, F_O (Karrasch and Walker, 1999; Orriss *et al.*, 1998; Rubinstein *et al.*, 2003).

Mammalian complex V contains at least 16 different subunits, of which two are encoded in the mtDNA (Collinson *et al.*, 1994; Lutter *et al.*, 1993). The F_1 assembly is a spherical complex containing the α and β subunits in a 3:3 stoichiometric ratio, penetrated by a central stalk of three single-copy subunits δ, ε, γ. It is the rotation of the central stalk along its long axis within the $\alpha\beta$ assembly that drives ATP synthesis (Gibbons *et al.*, 2000). The foot of the central stalk associates with the F_O assembly, which is comprised of a ring of 10-14 c-subunits (Pänke *et al.*, 2000; Sambongi *et al.*, 1999; Stock *et al.*, 1999; Tanabe *et al.*, 2001). The number of c-subunits in the F_O ring varies from species to species (Bottcher and Graber, 2000; Seelert *et al.*, 2000). The rotation of the c-ring and the central stalk ensemble is driven by the passage of protons through a channel between the c-ring and subunit a, encoded by the *ATP6* gene found on the mtDNA (Fillingame and Dmitriev, 2002). A peripheral stalk comprised of eight distinct subunits: OSCP (oligomycin sensitive conferral protein), b, d, e, f, g, A6L and F_6 links the F_O and F_1 assemblies (Collinson *et al.*, 1994; Collinson *et al.*, 1996). A6L is encoded by the *ATP8* gene, which is also found in the mtDNA. Single copies of subunits b, d, and F_6 are confirmed in the peripheral stalk, but the stoichiometries of the other components have yet to be determined. The peripheral stalk functions as a stator to hold the $\alpha_3\beta_3$ subcomplex and the peripheral membrane domain in place relative to the rotating c-ring and central stalk (Collinson *et al.*, 1996). The final component of complex V, the IF_1 subunit, is a regulatory inhibitor of the F_1 assembly that docks to the β-subunit.

1.4 Mitochondrial Biology

1.4.1 Reactive Oxygen Species

Mitochondria are directly involved in two cellular processes closely associated with mitochondrial diseases: the generation of reactive oxygen species (ROS) and apoptosis (discussed in subsection 1.4.2). Mitochondria are the major sites of ROS generation and consequently are the first cellular compartment to suffer damage by them. An estimated 1-2% of the oxygen consumed by the MRC is not fully reduced to water but instead is partially reduced to reactive oxygen intermediates such as the superoxide anion ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2). Superoxide can be converted to hydrogen peroxide by the manganese superoxide dismutase (MnSOD) and Cu/ZnSOD, which reside in the mitochondrial matrix and IMS, respectively. Hydrogen peroxide is a more stable and membrane-permeable species that can subsequently be converted to an even more reactive species, the hydroxyl radical ($OH^{-}\cdot$). ROS are responsible for a number of damaging effects such as lipid peroxidation, protein oxidation, DNA damage and diminished electron transport capacity and ATP synthesis. Under normal conditions, ROS are detoxified by mitochondrial MnSOD, cytosolic SOD and glutathione peroxidase (Poyton and McEwen, 1996). However, under conditions of cellular stress such as MRC dysfunction, ROS production can overwhelm these detoxification mechanisms and trigger cell death.

The principal sources of ROS generation within the MRC are thought to be complexes I and III, particularly under conditions of high membrane potential (Korshunov *et al.*, 1997). The 20-kDa PSST subunit of complex I was identified as the most likely site of ubisemiquinone generation and superoxide production (Schuler *et al.*, 1999). The redox cycling of ubiquinone in the Q-cycle of complex III has the potential to generate the most ROS (Cadenas *et al.*, 1977). When mutated, complex II can be a site of significant superoxide generation through flavin- or quinone-mediated events (Guo and Lemire, 2003; Ishii *et al.*, 1998; Senoo-Matsuda *et al.*, 2001). ROS production contributes to pathogenesis in a number of neurodegenerative diseases (Halliwell, 1992a; Halliwell, 1992b). Relevant to the studies presented in this thesis is the observation that patients with inherited complex I disorders have significantly elevated levels of MnSOD (Luo *et al.*, 1997; Pitkänen and Robinson, 1996; Robinson, 1994).

1.4.2 Apoptosis

Apoptosis or programmed cell death is a normal cellular process required for development, organ morphogenesis, aging and the removal of damaged cells (Joza *et al.*, 2001). Mitochondria are central regulators of apoptosis. The cytological features of apoptosis include cell shrinkage, loss of plasma membrane integrity and blebbing, chromatin condensation, DNA fragmentation and permeabilization of the mitochondrial outer membrane. The latter feature initiates most forms of apoptosis and is referred to as the mitochondrial pathway. Mitochondrial permeabilization can occur via two mechanisms. The first involves the Bcl-2 family of proteins that are recruited to the surface of mitochondria following an apoptotic stimulus (Finkel, 2001). By mechanisms that have yet to be determined, proapoptotic members of this family (Bid, Bax, Bak) permeabilize the mitochondrial outer membrane (Epand *et al.*, 2002a; Epand *et al.*, 2002b; Kudla *et al.*, 2000), allowing the release of cytochrome *c*, apoptotic inducing factor (AIF) and Smac/DIABLO from the intermembrane space to the cytosol. Other factors also appear to contribute to mitochondrial permeabilization. Bax appears to colocalize with the fission- and fusion-related proteins Drp1 and Mfn2 on the mitochondrial outer membrane, inducing mitochondrial fragmentation during apoptosis (Frank *et al.*, 2001; Karbowski *et al.*, 2002) and suggesting a relationship between apoptosis and the mitochondrial fission/fusion machinery.

The second mechanism of outer membrane permeabilization involves the formation of a proteinaceous complex called the mitochondrial permeability transition pore (mPTP), which joins the inner and outer membranes (Zamzami and Kroemer, 2001). The mPTP is believed to consist of the inner membrane proteins ANT and cyclophilin D and the outer membrane voltage-dependent anion channel (VDAC) and benzodiazepine receptor (Newmeyer and Ferguson-Miller, 2003). Sustained opening of the mPTP equilibrates ion concentrations between the matrix and the cytosol, dissipates the membrane potential, blocks respiratory function and protein import into the matrix and mediates the release of cytochrome *c* and other soluble proapoptotic factors of the intermembrane space. Ion redistribution leads to swelling of the matrix space and rupture of the outer membrane (Scorrano *et al.*, 2002).

Following outer membrane disruption, released cytochrome *c* recruits apoptotic peptidase activating factor 1 (Apaf-1), inducing its oligomerization and forming the apoptosome complex (Wang, 2001). The apoptosome recruits the proteinase caspase-9, which cleaves and activates caspases-3 and -7, commonly known as executioner caspases. Activated executioner caspases subsequently cleave a number of intracellular substrates leading to cell death. Interestingly, some of these intracellular targets include protein subunits of the MRC, specifically subunits of complexes I and II (Ricci *et al.*, 2003). The 75-kDa complex I subunit was recently identified as a caspase-3 substrate

(Ricci *et al.*, 2004). Apoptosis and the MRC-mediated generation of ROS are intimately related processes because ROS can either promote or inhibit apoptosis (Kirkland and Franklin, 2003; Van Antwerp *et al.*, 1996).

1.5 Mitochondrial Diseases

1.5.1 Respiratory Chain Disorders

Any defect that disrupts mitochondrial function or homeostasis can, in theory, cause a mitochondrial disorder. The vast majority of mitochondrial diseases results from defects in the MRC but disease-causing mutations are also found in nuclear genes affecting mitochondrial protein import (Koehler *et al.*, 1999; Roesch *et al.*, 2002), iron transport (Allikmets *et al.*, 1999; Campuzano *et al.*, 1996), copper transport (Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999; Valnot *et al.*, 2000a), mitochondrial maintenance (Delettre *et al.*, 2002), mtDNA copy number maintenance (Marin-Garcia *et al.*, 2000) and the ANT (Jordens *et al.*, 2002). I will focus my discussion of mitochondrial diseases on disorders stemming from MRC dysfunction. MRC dysfunction can affect any organ or tissue, at any age, and with any mode of inheritance due to the two genomes involved. The involvement of two genomes can make mitochondrial disorders difficult to diagnose and treat. Mitochondrial diseases are often multisystemic and/or tissue-specific, primarily affecting those tissues, such as the central and peripheral nervous systems, muscle, cardiac, renal, hepatic and endocrine tissues, with the greatest demand for OXPHOS (Munnich and Rustin, 2001). There is a tenuous link between genotype and phenotype; identical mutations can cause clinically distinct disorders or alternatively, mutations in different genes can cause the same disease.

The following is a list of some of the more common MRC disorders with brief descriptions to illustrate the diversity and tissue-specificity of these diseases. Further discussion on the specific nuclear and mitochondrial mutations that cause MRC disorders is presented in subsections 1.5.2 and 1.5.3.

Leigh syndrome: Leigh syndrome is an early-onset mitochondrial disorder that is frequently lethal. It is often characterized by psychomotor regression, leukodystrophy and brainstem dysfunction (Valanne *et al.*, 1998). Patients often present with ataxia, hypotonia, spasticity and developmental delay (Wallace *et al.*, 1998). The primary hallmark of the disease is the presence of spongiform lesions attributed to demyelination of neuronal axons. The disease is commonly caused by nuclear mutations in complexes I, II or IV, or in the pyruvate dehydrogenase complex. It can also be caused by mtDNA mutations (Kirby *et al.*, 2000; Makino *et al.*, 2000; Rahman *et al.*, 1996; Triepels *et al.*, 2001b; Zhu *et al.*, 1998).

Kearns-Sayre syndrome (KSS): KSS is a multisystemic disorder characterized by ophthalmoplegia, droopy eyelids, retinal degeneration leading to blindness and cerebellar ataxia with an onset before the age of 20. KSS is often a result of large-scale deletions or insertions in the mtDNA (Moraes *et al.*, 1989).

Leber hereditary optic neuroretinopathy (LHON): LHON is characterized by rapid vision loss due to optic nerve death, usually between the ages of 20 and 24 but also occurring at any age between adolescence and late adulthood (Munnich and Rustin, 2001). LHON is often associated with heart dysrythmia. It is the result of mtDNA mutations (DiMauro and Schon, 2001).

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS): MELAS is an early onset disorder characterized by exercise intolerance, recurrent neurological impairment resembling strokes, vomiting, lactic acidosis and myopathy with ragged red fibers. The disease is most often associated with a mutation in a mitochondrial tRNA gene (Goto *et al.*, 1990; McKenzie *et al.*, 2004).

Myoclonus epilepsy with ragged red fibers (MERRF): MERRF, another early onset disorder usually appearing before adolescence, is associated with epileptic episodes, ataxia, hearing loss, muscle weakness and the presence of ragged red fibers in muscle biopsies. Mutations are acquired sporadically or through the maternal inheritance of a mutation in a mitochondrial tRNA gene (Shoffner *et al.*, 1990; McKenzie *et al.*, 2004).

Neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP): In addition to the symptoms for which it is named, NARP is often associated with variable sensory neuropathy, seizures and mental retardation. The disorder is caused by a mtDNA mutation (Holt *et al.*, 1990; Munnich and Rustin, 2001).

Pearson syndrome: Pearson syndrome is a complex disorder characterized by anemia and pancreatic dysfunction, beginning in early infancy and often fatal before the age of three (Rötig *et al.*, 1995; Rötig *et al.*, 1990). Patients who do recover from the initial symptoms often develop KSS. The disease is a result of large-scale deletions and duplications of the mtDNA.

Progressive external ophthalmoplegia (PEO): PEO is associated with progressive muscle weakness and ophthalmoplegia. PEO patients can also present with ataxia or become comatose due to keto-acidosis. The disease is often caused by single or multiple mtDNA deletions (Moraes *et al.*, 1989; Zeviani *et al.*, 1989) that result when ANT mutations cause abnormal nucleotide availability for mtDNA synthesis (Kaukonen *et al.*, 2000).

Neurodegenerative disorders: MRC dysfunction is often associated with numerous neurodegenerative disorders. Huntington's disease is an autosomal dominant disorder associated with degeneration of striatal neurons and the cerebral cortex. The condiiton is caused by a CAG repeat expansion in huntingtin, a protein of unknown function. Huntington's disease patients often have deficiencies in complexes II and III in the basal ganglia (Browne *et al.*, 1997; Gu *et al.*, 1996). Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disorder affecting the spinal cord and cortical motor neurons, often associated with mitochondrial abnormalities (Orth and Schapira, 2001). Parkinson's disease is a late-onset disorder in which patients typically present with tremors and rigidity caused by loss of dopaminergic neurons in the substantia nigra of the brain. Individuals with Parkinson's disease often have a complex I deficiency, increased ROS production and in some cases, mtDNA mutations (Kösel *et al.*, 1999; Simon and Johns, 1999; Thyagarajan *et al.*, 2000). Finally, Alzheimer's disease, a mostly sporadic disorder that causes late-onset dementia is often associated with deficiencies in complex II and IV (Mutisya *et al.*, 1994; Ojaimi *et al.*, 1999; Parker and Parks, 1995).

1.5.2 Mitochondrial DNA Mutations

The majority of pathogenic MRC mutations has been identified in the mitochondrial genome. Diagnosing and treating these mutations can be more complex than for nuclear mutations because of their mode of inheritance and of the distinct properties of the mitochondrial genome. mtDNA is maternally inherited and cells typically contain hundreds to thousands of copies, usually of one genotype, which is referred to as a state of homoplasmy. Mutations arising in the mitochondrial genome can result in the coexistence of two populations within a single cell or tissue, one mutant and one wild-type, a state referred to as heteroplasmy. The proportion of mutated DNA, referred to as the mutational load, can affect the severity of the defect, often requiring a threshold level to be reached before symptoms are manifested (Chinnery *et al.*, 1999).

The random inheritance of mtDNA during cell division can result in altered mutational loads between cells in a tissue. The energy requirements of a cell- or tissue-type contribute to the tissue-specificity of mitochondrial disorders because tissues with the greatest demand for ATP, such as brain, muscle and heart are more easily compromised (Chinnery and Turnbull, 1997; Larsson *et al.*, 1998). Therefore, the severity of a disorder of origin in the mtDNA is influenced by the level of heteroplasmy and by the tissue distribution, making the linkage of genotype to phenotype extremely difficult.

mtDNA mutations can produce deficiencies in individual MRC complexes if they are located in one of the 13 MRC subunit genes or they can affect the transcription and translation of all 13 genes if the mutation is within a tRNA or rRNA gene. Two classes of pathogenic mtDNA mutations have been identified. The first, large-scale deletions, are causative of KSS, PEO and Pearson syndrome. Often associated with these deletions are mtDNA duplications, the presence of which may be linked to the mechanism of deletion formation (Samuels *et al.*, 2004). Point mutations make up the remainder of mtDNA lesions. Over 100 pathogenic point mutations have been identified (DiMauro and Andreu, 2000; Sue *et al.*, 2000); the majority is located within genes encoding tRNAs, thereby affecting the expression of all 13 mtDNA-encoded polypeptides.

The most common maternally-inherited mtDNA point mutations are in the $\mathrm{tRNA}^{\mathrm{Lys}}$ gene, producing MERRF and in the $\mathrm{tRNA}^{\mathrm{Leu(UUR)}}$ gene, producing MELAS when present in a high mutational load (>85%) or maternally-inherited diabetes mellitus and deafness when present at lower levels (5-30%) (Gerbitz *et al.*, 1995; Schon, 2000). The $\mathrm{tRNA}^{\mathrm{Leu(UUR)}}$ mutation offers a good example of difficulties encountered in diagnosing mitochondrial disorders. Other tRNA gene mutations are associated with PEO, myopathy, cardiomyopathy, encephalomyopathy and Leigh syndrome (Schon, 2000). The most common rRNA mutations, those occurring in the 12S rRNA, often cause aminoglycoside-induced, nonsyndromic hearing loss (Fischel-Ghodsian, 1999).

Point mutations in individual MRC subunit genes have also been identified (Figure 1.5). Of the ten pathogenic mutations in the ND genes, six are associated with LHON (Chalmers and Schapira, 1999). The others produce dystonia and sporadic myopathy (Schon, 2000). The twelve pathogenic mutations found in the cytochrome *b* gene were all sporadic rather than inherited and caused isolated myopathies (DiMauro and Andreu, 2000). Sporadic mutations in the mtDNA-encoded subunits of complex IV are linked to a series of unrelated disorders such as myopathy, encephalomyopathy, and anemia (Schon, 2000). Mutations in the *ATP6* gene of complex V are associated with striatal necrosis syndromes (Schon *et al.*, 1997). Furthermore, depending on the mutational load and the tissue distribution, the same *ATP6* mutation can produce maternally-inherited Leigh syndrome or the clinically-distinct disorder NARP.

1.5.3 Nuclear DNA Mutations

mtDNA mutations and deletions only account for 10-15% of cases of MRC dysfunction (Munnich and Rustin, 2001). Disorders associated with nuclear mutations often follow the traditional Mendelian mode of inheritance. Pathogenic nuclear mutations are typically of two classes: 1) mutations in genes encoding MRC structural subunits and 2) mutations in genes encoding MRC assembly and maintenance factors. Of the first class, mutations have only been identified in genes for complexes I and II despite extensive sequence analysis of many more MRC genes (Figure 1.5). The bulk of these are in the complex I genes *NDUFV1*, *NDUFS1*, *NDUFS2*, *NDUFS4*, *NDUFS7* and

NDUFS8. They cause a myriad of disorders including leukodystrophy, myoclonic epilepsy, cardiomyopathy, encephalomyopathy and Leigh syndrome (Bénit *et al.*, 2001; Loeffen *et al.*, 2001; Schuelke *et al.*, 1999; Triepels *et al.*, 2001a; van den Heuvel *et al.*, 1998). Pathogenic mutations in the *SDHA* gene of complex II are associated with Leigh syndrome (Bourgeron *et al.*, 1995; Parfait *et al.*, 2000). Mutations in the *SDHA*, *SDHC* and *SDHD* genes are associated with hereditary paraganglioma (Astuti *et al.*, 2001).

The second class of pathogenic nuclear mutations indicates that the assembly and maintenance of MRC complexes is as important as the integrity of their structural subunits. Six complex IV assembly factors are known (Figure 1.5): SURF1, a protein whose specific function remains unknown (Tiranti *et al.*, 1995; Zhu *et al.*, 1998), COX10, a heme A:farnesyl transferase (Valnot *et al.*, 2000b), COX15, which functions in the synthesis of heme A (Antonicka *et al.*, 2003), the leucine-rich pentatricopeptide repeat cassette (LRPPRC; Xu *et al.*, 2004), which plays a role in the translation or stability of mtDNA-encoded COX subunits, and SCO1 (Valnot *et al.*, 2000a) and SCO2 (Papadopoulou *et al.*, 1999), which are required for copper insertion. Mutations in these assembly factors produce distinct clinical syndromes. *SURF1* defects are associated with Leigh syndrome (Poyau *et al.*, 1999; Tiranti *et al.*, 1999; Tiranti *et al.*, 1998; Zhu *et al.*, 1998), *COX10* lesions with encephalomyopathy (Valnot *et al.*, 2000b), *COX15* defects with early-onset fatal hypertrophic cardiomyopathy (Antonicka *et al.*, 2003), mutations in the LRPPRC gene result in Leigh syndrome French Canadian (Xu *et al.*, 2004), *SCO1* mutations with neonatal liver failure and encephalomyopathy (Valnot *et al.*, 2000a) and *SCO2* defects with infantile cardiomyopathy (Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999). A defect in the complex III assembly factor BCSL1 is associated with tubulopathy, encephalomyopathy and liver failure (de Lonlay *et al.*, 2001).

1.5.4 Treatment of Mitochondrial Diseases

Although no categorical cure for mitochondrial disorders yet exists, there are therapeutic approaches that are used clinically to treat patients. Palliative treatment is a protective therapy that does not address the cause of the disorder but rather tries to ease the symptoms (Schon and DiMauro, 2003). Palliative treatment can include aerobic exercise to prevent muscle deconditioning and maintain function, surgeries to correct eyelid droopiness or cataracts, hearing aids to treat neurosensory hearing loss or the administration of pharmaceuticals to treat heart irregularities, diabetes or renal dysfunction.

Pharmacological treatments are administered to circumvent MRC defects. Some pharmacological supplements are used to specifically remove noxious metabolites. For example, a block in the respiratory chain can result in the accumulation of pyruvate in the cytosol, resulting in a subsequent increase in its reduction product lactate. Lactate build-up in the blood of patients can cause lactic acidosis, a common and severe symptom of mitochondrial disorders; elevated lactate in the central nervous system is a neurotoxin. Anecdotal evidence has demonstrated that supplements like dichloroacetate (DCA), which inhibits the phosphorylation of the pyruvate dehydrogenase complex (PDHC) and keeps the enzyme in the active state, can improve the clinical outcome of disorders involving lactic acidosis (Chinnery and Turnbull, 2001; Schon and DiMauro, 2003; Taylor *et al.*, 1997). The same has been observed for thiamine, also an activator of PDHC, when given in combination with DCA (Stacpoole *et al.*, 1998).

Some pharmacological supplements can be used to bypass biochemically the block in electron transport. For instance, a combination of ascorbate (vitamin C) and menadiol diphosphate, a precursor of vitamin K was administered to a patient with an isolated complex III deficiency. The supplements were used as artificial electron acceptors to bypass the defect in complex III. The results of the treatment showed dramatic, yet unsustained improvements in the patient's symptoms (Eleff *et al.*, 1984). Other supplements have been used to enhance cofactor and metabolite concentrations in patients. For example, riboflavin, a precursor of the FMN cofactor of complex I, benefited patients with complex I deficiency and mitochondrial myopathy due to mutation in the $tRNA^{Leu(UUR)}$ (Ogle *et al.*, 1997). Finally, some supplements have been used to reduce the damage caused by the excess generation of ROS. These include treatment with free-radical scavengers such as coenzyme Q_{10} (CoQ_{10}) and idebenone, a quinone compound similar to CoQ_{10} (Abe *et al.*, 1999; Delanty and Dichter, 2000).

The successful identification of genes responsible for mitochondrial disorders has promoted the development of treatments involving gene therapy strategies. Many of these approaches involve the replacement or addition of gene products to directly correct or bypass the defect. Some strategies have taken advantage of simpler respiratory enzymes from other species in order to bypass MRC defects in mammalian cells. This strategy, referred to as allogenic gene therapy, was utilized to circumvent a complex I deficiency in mammalian cells using the *S. cerevisiae* internal NADH-quinone oxidoreductase Ndi1p (Seo *et al.*, 1998). This single-subunit enzyme serves as the primary entry-point of electrons into the yeast MRC; the organism does not possess a mammalian-like complex I. The yeast *NDI1* gene was successfully transfected into complex I-deficient Chinese hamster cells, resulting in the functional expression of the enzyme and restoration of NADH oxidation. Similar results were observed in the successful and efficient transfection of cultured human cell with *NDI1* (Seo *et al.*, 1999; Seo *et al.*, 2000). Recently, this strategy was extended by demonstrating the sustained *in vivo* expression of *NDI1* in the skeletal muscle and brains of rodents (Seo *et al.*, 2004). Ndi1p has also been shown to complement a defect in human cells harbouring a mutation in the mtDNA-encoded *ND4* gene (Bai *et al.*, 2001). The early successes of these investigations clearly show that allogenic gene therapy has the potential to be a lasting molecular remedy for complex I deficiency and that additional 'simple' respiratory genes could be utilized to bypass defects in other MRC complexes.

Gene therapy approaches for mtDNA mutations is more complicated. Currently, it is technically difficult to transfect mammalian mitochondria with exogenous DNA. However, it is more feasible to express a mtDNA-encoded gene in the nucleus. This strategy is referred to as allotopic gene expression (Gray *et al.*, 1996) and has been successfully applied to cultured human cells (Manfredi *et al.*, 2002). Human cells homoplasmic for the T8993G NARP/MILS mutation in the *ATP6* gene showed improved growth under selective conditions and increased ATP synthase activity when allotopically transfected with a recoded (for nuclear expression), functional *ATP6* gene (Manfredi *et al.*, 2002). A similar strategy was applied to cells carrying mutations within the mtDNA-encoded *ND4* gene, which causes maternally-inherited LHON. Allotopic expression of human *ND4* in these cells was able to complement the defect (Guy *et al.*, 2002). Other studies have utilized nuclear DNA-encoded tRNAs targeted to the mitochondrion to correct mutations in mtDNA-encoded tRNAs. This approach has been successfully applied to patient-derived cell cultures bearing a $tRNA^{Lys}$ mutation responsible for MERRF (Kolesnikova *et al.*, 2004). Finally, there is recent work that addresses new technology for the direct transfection of DNA into mitochondria. 'Protofection' is the protein transduction domain (PTD)-mediated insertion and expression of human mitochondrial genomes into the mitochondria of living cells, allowing for the alteration of mtDNA genotype or the expression of exogenous genes (Khan and Bennett, 2004). PTDs are small regions of protein that are able to cross membranes in a receptor-independent fashion. Although the technology appears promising, little has been published about it and the precise methodology has yet to be revealed.

1.5.5 Higher Model Systems

Therapeutic intervention to improve or cure MRC diseases is hindered by our poor understanding of key pathogenic events. The limited availability of human tissue makes it necessary to study the molecular details of pathogenesis in model organisms. For some pathological studies, the profound differences between humans and lower model organisms prevent the generation of significant and relevant information. For this reason, substantial work on mitochondrial disorders has been done in the mouse model. In many cases, mice accurately reproduce human disease pathology and the organ-specific patterns of MRC deficiency. Mouse models now exist for a myriad of mitochondrial dysfunctions, including a knock-out of the *FRDA* gene involved in Friedrich's ataxia (Cossee *et al.*, 2000; Puccio *et al.*, 2001), tissue-specific knockouts of *Tfam*, which result in mtDNA depletion and MRC deficiency (Larsson *et al.*, 1998; Li *et al.*, 2000; Silva *et al.*, 2000; Wang *et al.*, 1999), large mtDNA deletions, resulting in MRC deficiency (Inoue *et al.*, 2000), and defects in the DNA polymerase γ, which allow for the accumulation of mtDNA mutations that promote apoptosis and may serve as a mammalian model for aging (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). The mouse model does suffer some serious disadvantages for the study of mitochondrial diseases. Mouse studies are often time- and labour-intensive. The mice themselves are cumbersome and expensive to maintain and are not highly amenable to genetic manipulation. Finally, mice differ significantly from humans in some of their metabolic pathways and in their less developed brain functions.

The study of mitochondrial disorders has been greatly enhanced with the development of cytological hybrids, or cybrids. These can be formed by fusing an enucleated cell containing mitochondria carrying wild-type or mutant mtDNA to another cell depleted of mtDNA (ρ^0). Single cells with common nuclear backgrounds but different mtDNA compositions can be created, cultured and maintained. Nuclear mutations can be studied in a similar fashion by fusing a ρ^0 cell containing the mutation to an enucleated cell that provides wild type mtDNA. Cybrids have been extensively used in the study of mitochondrial dysfunction in neurodegenerative disorders (Trimmer *et al.*, 2000), large-scale mtDNA deletions (Inoue *et al.*, 2000) and gene therapy for the treatment of clinical disorders (Zullo *et al.*, 2005). The major advantage of cybrids is that it allows for the study of both nuclear and mtDNA mutations at the biochemical and genetic levels. However, the bulk of cybrid investigations has been done in cell culture, which does not address the issues of tissue-specificity and genetic transmission and may not accurately represent the pathogenic metabolism that occurs *in vivo*.

1.6 *Caenorhabditis elegans*

1.6.1 C. elegans Model System

Caenorhabditis elegans is a small, free-living soil nematode. In the mid-1960's, Sydney Brenner selected it as a simple model animal system for investigations into the genetics and development of the nervous system (Brenner, 1974). Today, our knowledge of *C. elegans* biology is extensive and exceeds that of many other model systems developed before it. *C. elegans* is a ravenous organism that primarily feeds on bacteria in a laboratory environment (Lewis and Fleming, 1995). It has a relatively rapid life-cycle of ~3 days under optimal conditions (Riddle *et al.*, 1997). The nematode exists in two sexes, hermaphrodite and male, which grow to ~1 mm in length at adulthood, but differ in their anatomy and appearance. Hermaphrodites (Figure 1.6) produce both oocytes and sperm and can reproduce by self-fertilization or by cross-fertilization with a male; hermaphrodites cannot fertilize each other. A self-fertilizing hermaphrodite typically lays ~300 eggs during its reproductive life span. *C. elegans* embryos hatch and normally develop through four larval stages of development (referred to as L1 to L4 stages; Figure 1.7). Larval stages are separated by molts and distinguishable from each other by progressive increases in size and numbers of germ line cells. Germ line cells in the hermaphrodite differentiate into sperm at the late L4 stage and into oocytes during early adulthood (Schedl, 1997). Maturation of the hermaphrodite reproductive system occurs primarily at the L4 stage. Under conditions of stress or food deprivation, the developing nematode can enter an alternative life-stage after L2, called the dauer stage. Dauer larvae can remain dormant for months, but when conditions become favourable again, a *C. elegans* larva can exit this stage and resume development to adulthood from the L4 stage

(Riddle and Albert, 1997). A mature hermaphrodite is fertile for about four days and total life span typically varies from 10-20 days.

C. elegans is both an anatomically and genetically simple organism. An adult hermaphrodite has exactly 959 somatic nuclei, of which 302 are neurons; the adult male has 1031 somatic nuclei. The nematode contains multiple tissue types such as neurons, muscle, intestine and germ-line (Wood, 1988), and its transparency makes the morphology of the organism easily examinable under the microscope. The haploid genome size of the organism is ~1.0 x 10^8 base pairs; it is the first multicellular eukaryote to have its entire genome sequenced (*C. elegans* Sequence Consortium, 1998). The *C. elegans* mitochondrial genome, at 13,794 bp in size, has also been completely sequenced (Okimoto *et al.*, 1992). Nuclear genes in *C. elegans* can be mapped to six linkage groups corresponding to six haploid chromosomes, five autosomes (I – V) and a sex chromosome (X). Hermaphrodites are diploid for all six chromosomes (XX), while males are diploid for the five autosomes and have only one sex chromosome (XO). Hermaphrodites dominate a typical *C. elegans* population, as males arise spontaneously within the population by X-chromosome non-disjunction at a frequency of ~0.2%.

The use of *C. elegans* to study a diverse array of fundamental biological processes has proliferated to hundreds of laboratories over the last four decades. The organism has a number of key attributes that make it an ideal experimental system. First, *C. elegans* is easily and economically maintained in the laboratory where it can be grown on solid agar plates or liquid culture with the common laboratory bacterium *Escherichia coli* as a food source. Its relatively short life cycle and ability to reproduce by self-fertilization make it easy to cultivate. Second, the developmental fates, locations and characteristics of all somatic cells in the adult hermaphrodite and male are known. This includes the timing, locations and ancestral relationships of all cell divisions during development, making it possible to pinpoint the exact developmental consequences of specific mutations. Finally, the relatively small size of the nematode genome and the availability of the complete mtDNA and nuclear DNA sequences facilitate the use of both forward and reverse genetic approaches for investigating gene function. *C. elegans* is amenable to genomic DNA transformation via microinjection, which generates transgenic animals with extrachromosomal arrays (Mello and Fire, 1995), or via ballistic transformation, which can produce low-copy extrachromosomal arrays or integrated transgenes (Jackstadt *et al.*, 1999; Praitis *et al.*, 2001; Wilm *et al.*, 1999). The simplicity and elegance of the nematode system also makes it ideal for the design and execution of genetic screens (Jorgensen and Mango, 2002). Finally, the now popular phenomenon of RNA interference (RNAi), a method that generates loss-of-function phenocopies with double-stranded RNA, has been extensively studied and used in *C. elegans* (Fire *et al.*, 1998).

Completion of the *C. elegans* genome sequence and the subsequent alignment of the physical and genetic maps have stimulated the use of the nematode in the study of human disorders. A survey of the *C. elegans* genome revealed many genes with a high degree of similarity to human disease genes, suggesting that the basic functions of these gene products are likely conserved (Aboobaker and Blaxter, 2000). New insights into disease have been gleaned from the *C. elegans* system for spinal muscular atrophy, polycystic kidney disease, muscular dystrophy, and a number of neurodegenerative disorders (Culetto and Sattelle, 2000; Driscoll and Gerstbrein, 2003). In the last decade, *C. elegans* studies of MRC dysfunction and human mitochondrial diseases have gained prominence.

1.6.2 Mitochondrial Dysfunction in C. elegans

Studies detailing *C. elegans* mitochondrial function were pioneered in the mid-1970s (Murfitt *et al.*, 1976). Measurements of respiration rates and enzyme activities demonstrated that the structure, function and bioenergetics of the nematode MRC are similar to the mammalian MRC. Subsequent studies showed that many of the pathways of intermediary metabolism that involve mitochondria are present in *C. elegans* (O'Riordan and Burnell, 1989; Wadsworth and Riddle, 1989). A bioinformatic survey of the *C. elegans* nuclear and mitochondrial genomes revealed that the majority of nematode MRC subunits shares significant sequence identity with their mammalian homologues. At least 36 complex I subunits, four subunits of complex II, at least eight subunits of complex III, at least nine subunits of complex IV, and a minimum of 14 subunits of complex V have been identified in *C. elegans* (Tsang and Lemire, 2003b). The *C. elegans* mtDNA, encoding 12 highly-conserved mtDNA-encoded MRC subunits, is slightly smaller than its mammalian counterpart, missing the *ATP8* gene and having a different gene organization (Figure 1.8) (Okimoto *et al.*, 1992). The similarity of the worm MRC to the mammalian one, plus the experimental attributes of the nematode make it an extremely attractive model system for the study of mitochondrial dysfunction and disease manifestation. Although still in their infancy, mitochondrial studies in *C. elegans* have produced some significant findings, which I will summarize in the following paragraphs (Figure 1.9).

nuo-1, the primary gene of interest in this thesis, encodes the 51-kDa subunit of complex I, which carries the substrate binding site and flavin and iron-sulfur cofactors involved in electron transfer. The NUO-1 protein shares ~75% sequence identity with the human NDUFV1 protein. Nematodes carrying the *nuo-1(ua1)* deletion were generated using target-selected mutagenesis (Tsang *et al.*, 2001). The 1,190-bp deletion removes the first three exons and the beginning of the fourth, eliminating the mitochondrial targeting sequence at the N-terminus and the NADH- and FMN-binding sites. For these reasons, the *nuo-1(ua1)* allele is believed to be null. Animals homozygous for the deletion arrest at the L3 stage, a lethal phenotype since reproduction is impossible. Gonad development is more severely affected, arresting at an L2 stage. Homozygous *nuo-1(ua1)* nematodes have a number of behavioral defects including reduced mobility, impaired feeding, a slower defecation cycle and an increased life span. Furthermore, homozygous mutant animals are able to enter the dauer stage, but cannot exit it. The *C. elegans* nervous system appears to have the greatest requirement for MRC function because ectopic expression of *nuo-1* under a nervous system-specific promoter in a homozygous *nuo-1(ua1)* background supports development to the adult stage and allows dauer larvae to exit this stage and proceed with development (Ndegwa and Lemire, 2004). Specific expression of *nuo-1* in other tissues is less effective at supporting development beyond the L3 stage. These studies suggest that MRC function, particularly in the nervous system, is needed to send and/or receive signals that control larval development.

Target selected mutagenesis also identified a deletion in the *atp-2* gene, which encodes the β-subunit of the F_1 subcomplex of the ATP synthase (Tsang and Lemire, 2003b; Tsang *et al.*, 2001). The *atp-2(ua2)* deletion allele is a 710-bp deletion that removes the first exon and the beginning of exon two, thereby removing the mitochondrial targeting sequence; it is also believed to be a null allele. Nematodes homozygous for the *atp-2(ua2)* allele have a similar phenotype to that of the homozygous *nuo-1(ua1)* animals: larval arrest at L3 stage with an L2-stage gonad, significantly lengthened life span, decreased motility, impaired feeding and a slower defecation cycle. The only significant difference between the *nuo-1* and the *atp-2* phenotypes is that *atp-2(ua2)* nematodes are unable to enter dauer stage (Tsang and Lemire, 2003b; Tsang *et al.*, 2001). Larval arrest at the L3 stage is also observed when nematodes are exposed to drugs that inhibit mitochondrial replication, transcription or translation, indicating that MRC-generated energy is essential for development and that an energy sensing mechanism may be present (Dillin *et al.*, 2002; Tsang *et al.*, 2001). Mosaic analysis revealed that *atp-2* functions in a cell non-autonomous manner, suggesting that development past the L3 stage is controlled by a global mechanism. The loss of *atp-2* in any tissue could provoke arrest at the L3 stage, although loss of *atp-2* in the cell lineages that give rise to body muscle cells invariably caused developmental arrest, indicating that this tissue plays a key role in regulating development (Tsang and Lemire, 2003a).

A mutation in the *mev-1* gene was originally identified in a screen for hypersensitivity to methyl viologen (paraquat), a known generator of ROS (Ishii *et al.*, 1990). *mev-1* encodes the cytochrome *b* subunit (SDHC) of complex II. The *mev-1(kn1)* mutant contains a missense mutation resulting in a Gly71Glu substitution (Ishii *et al.*, 1998). *mev-1* mutants are hypersensitive to oxidative stress, age prematurely, have a decreased life span and are hypermutable, especially under hyperoxic conditions

(Hartman *et al.*, 2001). In *mev-1* animals, the ability of complex II to oxidize succinate is unaffected, but its ability to reduce ubiquinone in a succinate-dependent manner is severely compromised (Ishii *et al.*, 1998). Furthermore, *mev-1* mutants show indications of abnormal energy metabolism (Senoo-Matsuda *et al.*, 2001), display abnormal mitochondrial ultrastructure, have reduced mitochondrial membrane potential and suffer premature apoptosis (Senoo-Matsuda *et al.*, 2003). This mutant phenotype is primarily attributed to the increased generation of superoxide (Senoo-Matsuda *et al.*, 2001), implying a critical role for complex II in superoxide production. Finally, inactivation of three of the four subunits of complex II by RNAi results in embryonic lethality (Ichimiya *et al.*, 2002), further reinforcing the importance of the MRC in nematode development.

The *gas-1(fc21)* mutation was originally isolated in a screen for hypersensitivity to volatile anesthetics (Kayser *et al.*, 1999). The *gas-1* gene (K09A9.5) encodes one of two homologues of the 49-kDa subunit of complex I. The *fc21* allele introduces a missense mutation resulting in an Arg290Leu substitution. *gas-1(fc21)* nematodes are hypersensitive to inhalation anesthetics and to ethanol, have reduced life spans, slow growth rates and like *mev-1* mutants are hypersensitive to oxidative stress (Kayser *et al.*, 1999). Furthermore, the *gas-1(fc21)* allele is lethal at higher temperatures. Mutation in the *gas-1* gene severely reduces oxidative phosphorylation and electron transport, although an increase in complex II-dependent activity is observed (Kayser *et al.*, 2001). Unlike *mev-1* mutants, *gas-1* mutant nematodes are not hypermutable and do not overproduce superoxide anions (Hartman *et al.*, 2001). GAS-1 protein is widely expressed in the *C. elegans* neuromuscular system and has a subcellular localization indicative of a mitochondrial protein (Kayser *et al.*, 2001). Mutated GAS-1 likely causes misassembly of complex I, but the mechanism by which it contributes to anesthetic sensitivity remains unknown.

The *isp-1(qm150)* allele, affecting the iron-sulfur protein of complex III, introduces a Pro225Ser substitution, which is in close proximity to the iron-sulfur cluster and is believed to alter its redox properties (Feng *et al.*, 2001). *isp-1(qm150)* nematodes have slow embryonic development, life spans double that of wild-type animals, reduced oxygen consumption rates and reduced motility (Feng *et al.*, 2001; Murakami and Murakami, 2005). A spontaneous partial suppressor of the slow rate of embryonic development of *isp-1(qm150)* animals was mapped to the *ctb-1* gene of the mtDNA, which encodes the cytochrome *b* subunit of complex III. The *ctb-1(qm189)* allele is a homoplasmic maternally-inherited mutation changing Ala-170 to Val (Feng *et al.*, 2001). The *ctb-1* mutation does not have a phenotype on its own, although it increases respiration rates, resistance to paraquat and partially suppress the slow behavioral and developmental phenotypes of *isp-1* mutants without affecting the extended life span of these animals.

A number of other mutations have been identified in genes that affect MRC function but do not reside in genes for MRC subunits. The *dif-1* gene, which encodes a mitochondrial acylcarnitine carrier, was originally identified in a general screen for maternal-effect, embryonic lethal mutants (Ahringer, 1995; Oey *et al.*, 2005). The gene plays a role in tissue differentiation in the developing embryo but how this relates to its function as a mitochondrial carrier protein remains unclear. A mutation in the *lrs-2* gene encoding the mitochondrial leucyl-tRNA synthetase significantly extends life span, resulting in slowly developing L4-sized, sterile adults with arrested gonad development

(Lee *et al.*, 2003). The LRS-2 protein is required for the translation of the 12 mtDNA-encoded MRC genes. *lrs-2* mutants have abnormal mitochondrial morphology, reduced ATP contents and oxygen consumption rates and are hypersensitive to paraquat. The latter observation suggests that the extended life span is not due to a decrease in ROS production but rather to an alternative mechanism. A mutation in the *gro-1* gene, encoding a pentenyl pyrophosphate:tRNA transferase, which modifies a subset of mitochondrial tRNAs, results in extended life span, severely reduced fecundity and a deregulation of behavioral and developmental rates (Lemieux *et al.*, 2001). The *coq-3(qm188)* mutation is a deletion in the gene encoding a methyltransferase for the biosynthesis of ubiquinone (Hihi *et al.*, 2002). Homozygous *coq-3* mutant progeny born of a heterozygous hermaphrodite are able to reach adulthood but are usually sterile. Those that are able to reproduce have extremely low brood sizes and their progeny die as L1 larvae, indicating a need for ubiquinone during development. Finally, there have been extensive studies done on the *clk-1* gene, which encodes a mitochondrial protein related to the yeast Coq7p, another protein required for ubiquinone biosynthesis (Ewbank *et al.*, 1997; Wong *et al.*, 1995). Under normal growth conditions, *clk-1* mutations result in significant life span extension and deregulate development and metabolic timing, resulting in extended cell-cycle periods, reduced mobility and feeding and longer defecation cycles. However, when *clk-1* mutant animals are grown on ubiquinone-deficient bacteria, they arrest as larvae (Jonassen *et al.*, 2001). Over-expression of the CLK-1 protein in a wild-type background accelerates aging, increases mitochondrial activity, accelerates behavioral rates during aging and shortens life span, indicating that *clk-1* is a regulator of these processes (Felkai *et al.*, 1999). These and other investigations have led to the hypothesis that the CLK-1 protein or its product ubiquinone have functions beyond quinone synthesis and MRC function and may be part of an elusive mechanism that controls the aging process (Branicky *et al.*, 2000; Echtay *et al.*, 2000; Georgellis *et al.*, 2001).

1.7 Thesis Objective

In humans, mutations that impair MRC function contribute, by mechanisms that are not well understood, to the development of a diverse and complex set of disorders that remain largely untreatable. The study of MRC disorders and the linkage of genotype to phenotype are complex. The small number of available patients often limits clinical studies and biopsy material is not amenable to genetic manipulation. Although considerable insight into mitochondrial disease has been gained with traditional genetic and biochemical model systems such as yeast, *N. crassa*, fruit flies and the mouse, each of these systems has its own particular advantages and limitations. Currently none of these model systems allow for the investigation of all aspects of mitochondrial dysfunction. There is still a need to establish new model systems. *C. elegans* is a recent addition to the collection of model systems used in mitochondrial investigations. There is a substantial and rapidly growing body of work addressing mitochondrial dysfunction and the role of the organelle in other processes such as apoptosis and aging. Its genetic and biochemical amenability, easy and rapid cultivation, combined with its multisystemic, yet simple anatomy make *C. elegans* a prized system for studying biological processes. The highly conserved nature of nematode metabolism and MRC structure, function and bioenergetics offers numerous possibilities for the study of mitochondrial disorders.

Our understanding of the relationship between genotype and phenotype is incomplete. The work I present in this thesis will demonstrate that the *C. elegans* system can address this issue. Previous investigations involving nematode mitochondrial dysfunction utilized forward genetic approaches to identify mitochondrial genes from mutant phenotypes. This thesis presents studies based on a reverse genetic strategy in which pathogenic human mutations are modeled in *C. elegans* in order to determine the molecular mechanisms of disease. Chapter 2 describes the generation, isolation and systematic characterization of transgenic mutants expressing three independent missense mutations in the *nuo-1* gene of complex I. These mutations are modeled after pathogenic *NDUFV1* mutations causing myoclonic epilepsy, leukodystrophy, severe metabolic acidosis and Leigh syndrome (Bénit *et al.*, 2001; Schuelke *et al.*, 1999). This investigation addresses the molecular and metabolic effects of these three mutations and attempts to explain the observed phenotypes. The study also explores pharmacological treatment of mutant nematodes, not only as a means of therapy but also as a way to detect alterations in metabolic pathways and to modulate them.

While Chapter 2 addresses the functional consequences of complex I deficiency, Chapter 3 addresses the physical integrity of the MRC complexes in the mutant background. Western blot analysis and blue-native gel electrophoresis are used to determine whether catalytic deficiencies are a consequence of complex instability and/or of misassembly.

Although there are no cures for mitochondrial disorders, there is a small but growing body of work presenting gene therapy approaches to correct MRC deficiencies. The majority involves the use of transformation in an attempt to functionally replace defective MRC enzymes. Chapter 4 describes a novel gene therapy approach involving the yeast gene *CYB2*, which encodes an L-lactate-cytochrome *c* oxidoreductase not found in *C. elegans* or mammals. *CYB2* expression creates a new metabolic pathway in complex I-deficient *C. elegans* that bypasses the defect and corrects a metabolite imbalance caused by complex I dysfunction. The *CYB2* gene therapy strategy not only demonstrates new avenues for the treatment of mitochondrial disorders but it also sheds additional insight into the pathogenic mechanisms of complex I mutations.

The final chapter of the thesis discusses the future of mitochondrial disease research in the nematode. Many questions remain to be answered. What aspects of the pathogenic mechanisms of complex I-associated disease remain unexplained? Is there a future for the *C. elegans* model system in studying human mitochondrial disorders or other diseases? What aspects of mitochondrial dysfunction deserve additional attention? Will new technologies need to be developed to study them? What does the future hold in store for the treatment of mitochondrial diseases? These questions and others will hopefully provoke new and exciting investigations into mitochondrial diseases.

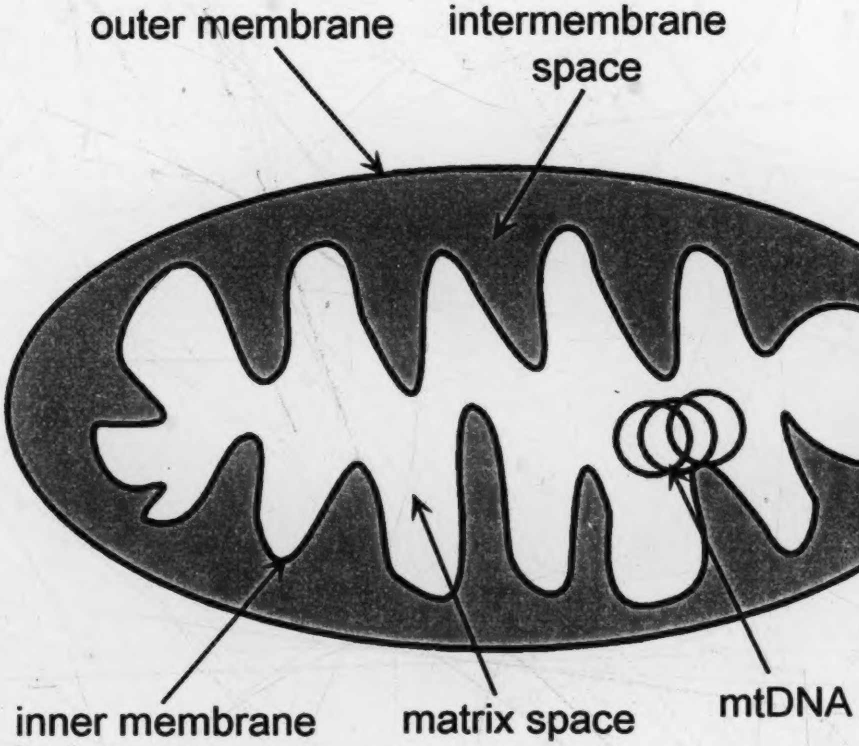
outer membrane
intermembrane space
inner membrane
matrix space
mtDNA

Figure 1.1 Schematic of mitochondrial ultrastructure.

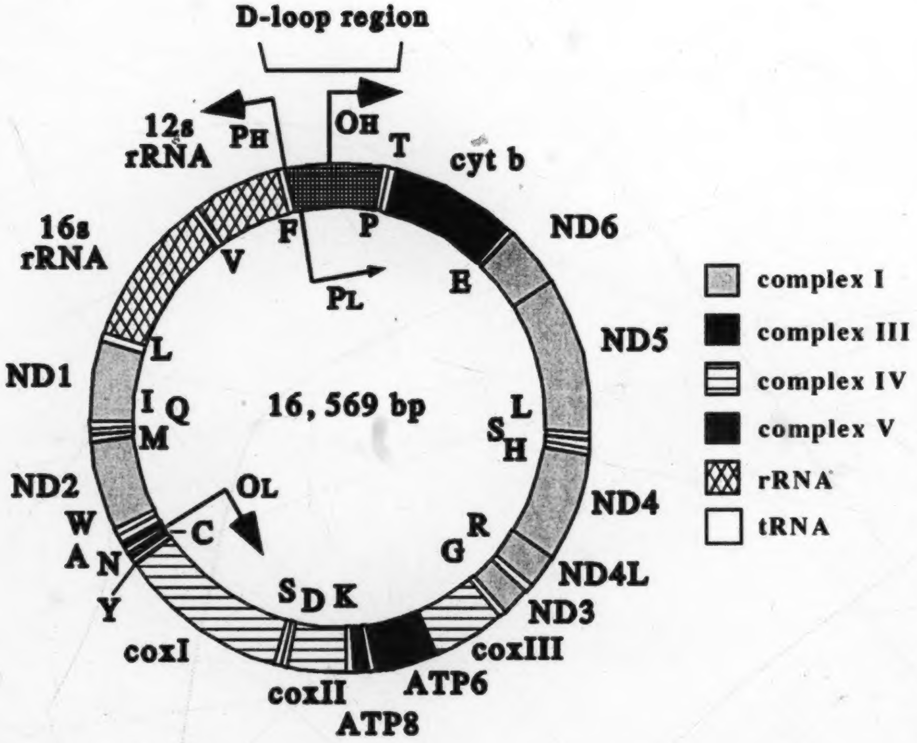
D-loop region
12s rRNA
PH
OH
T
cyt b
16s rRNA
F
P
V
PL
E
ND6
ND5
L
ND1
I
Q
M
16, 569 bp
L
S
H
ND2
OL
W
C
A
N
Y
R
G
ND4
ND4L
ND3
S
D
K
coxI
coxII
ATP8
ATP6
coxIII
complex I
complex III
complex IV
complex V
rRNA
tRNA

Figure 1.2 Circular map of the human mtDNA. tRNAs are identified by their one letter amino-acid code. P_L, light-strand promoter; P_H, heavy-strand promoter; O_L, light-strand origin of replication; O_H, heavy-strand origin of replication.

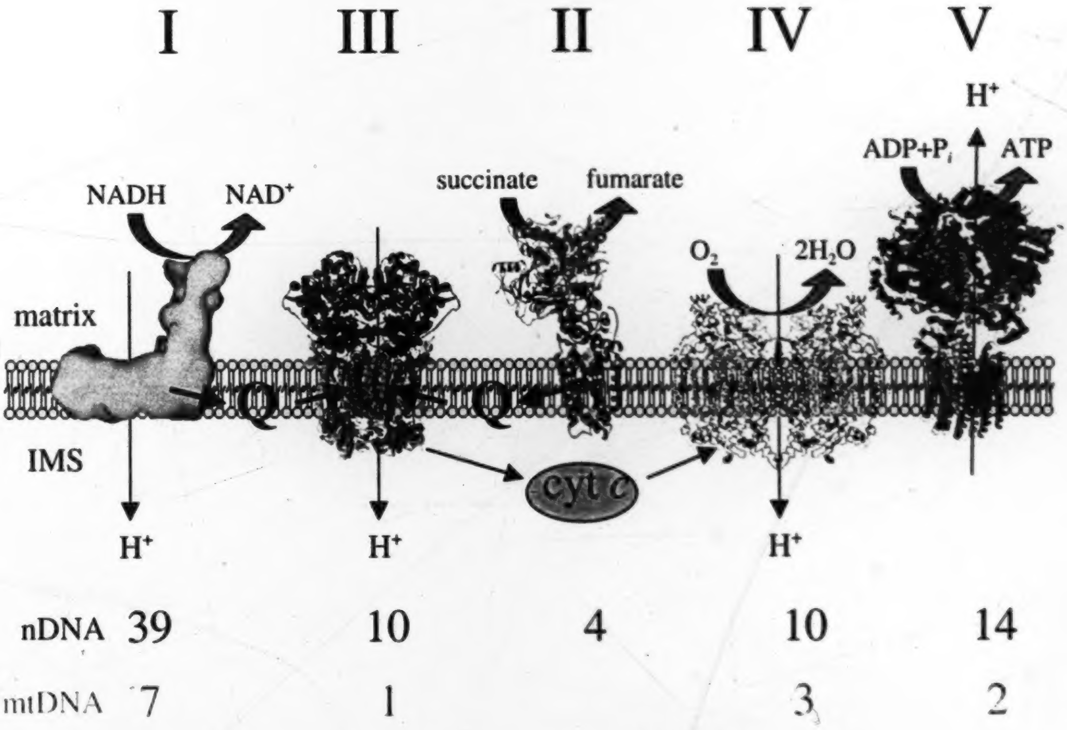
I
III
II
IV
V
H+
ADP+Pi
ATP
NADH
NAD+
succinate
fumarate
O2
2H2O
matrix
Q
Q
IMS
cyt c
H+
H+
H+
nDNA 39
10
4
10
14
mtDNA 7
1
3
2

Figure 1.3 Mammalian mitochondrial respiratory chain (MRC). MRC complexes are represented by their known three-dimensional structures. Black numbers indicate the number of nuclear-encoded subunits for each complex. Grey numbers indicate the number of mtDNA-encoded subunits for each complex. Q, ubiquinone; cyt *c*, cytochrome *c*, IMS, intermembrane space.

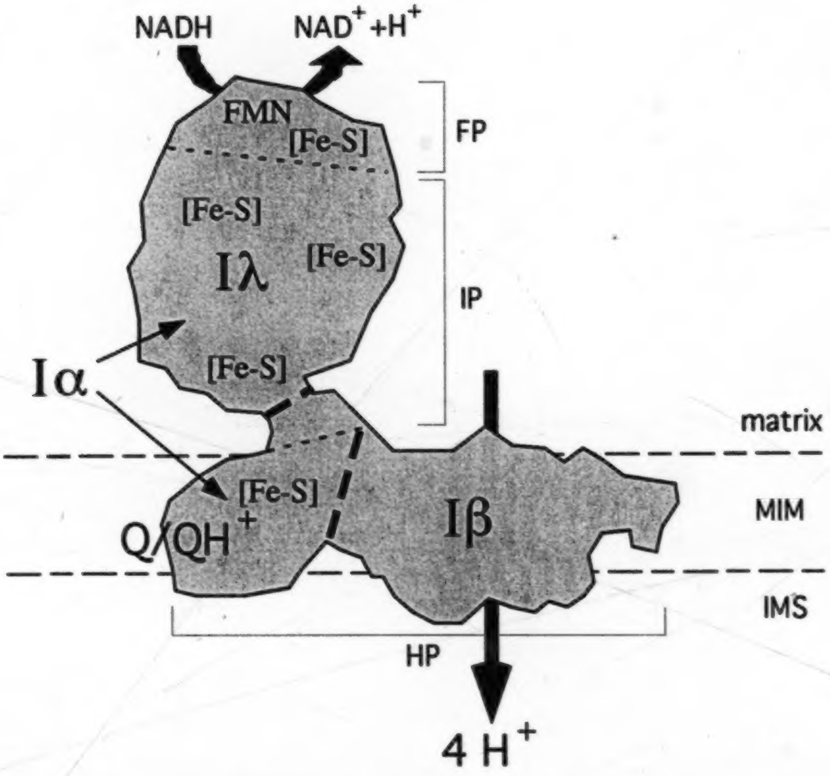
NADH
NAD$^+$+H$^+$
FMN
[Fe-S]
FP
[Fe-S]
Iλ
[Fe-S]
IP
Iα
[Fe-S]
matrix
[Fe-S]
Q/QH$^+$
Iβ
MIM
IMS
HP
4 H$^+$

Figure 1.4 Schematic of mammalian complex I. Schematic based on the three-dimensional structure of complex I from bovine heart (Grigorieff, 1998). Light dashed line indicates separation of complex I into FP (flavoprotein), IP (iron-sulfur protein), and HP (hydrophobic protein) subcomplexes. Heavy dashed line indicates separation of complex I into Iα, Iβ, and Iλ subcomplexes. [Fe-S], iron-sulfur cluster; Q/QH$^+$, oxidized/reduced ubiquinone; FMN, flavin mononucleotide, IMS, intermembrane space; MIM, mitochondrial inner membrane. Adapted from Hirst *et al.* (2003).

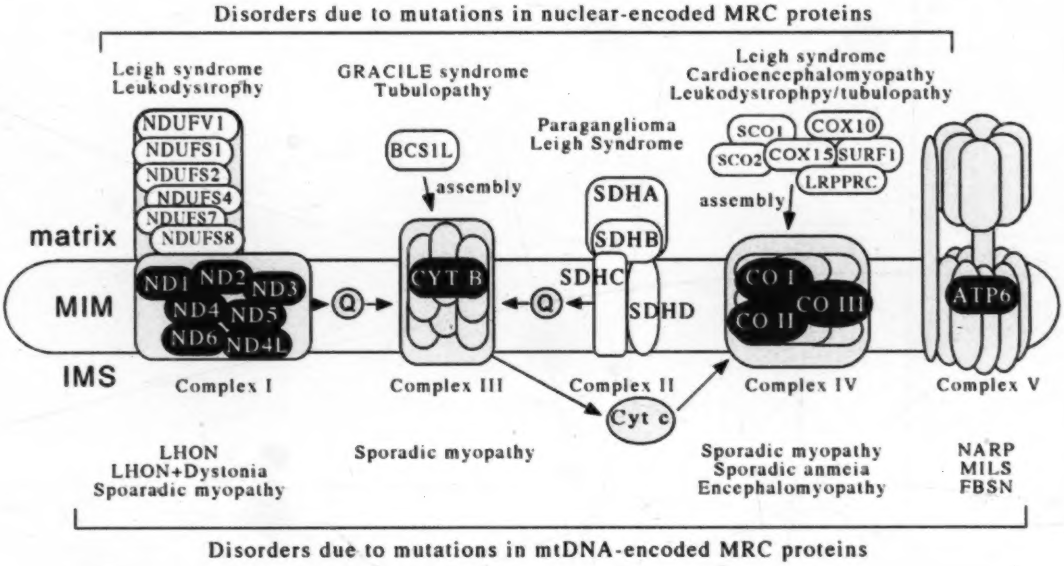
Disorders due to mutations in nuclear-encoded MRC proteins
Leigh syndrome
Leukodystrophy
GRACILE syndrome
Tubulopathy
Leigh syndrome
Cardioencephalomyopathy
Leukodystrophpy/tubulopathy
Paraganglioma
Leigh Syndrome
NDUFV1
NDUFS1
NDUFS2
NDUFS4
NDUFS7
NDUFS8
BCS1L
assembly
SDHA
SDHB
SDHC
SDHD
SCO1
COX10
SCO2
COX15
SURF1
LRPPRC
assembly
matrix
MIM
IMS
ND1
ND2
ND3
ND4
ND5
ND6
ND4L
Q
CYT B
Q
CO I
CO II
CO III
ATP6
Complex I
Complex III
Complex II
Complex IV
Complex V
Cyt c
LHON
LHON+Dystonia
Spoaradic myopathy
Sporadic myopathy
Sporadic myopathy
Sporadic anmeia
Encephalomyopathy
NARP
MILS
FBSN
Disorders due to mutations in mtDNA-encoded MRC proteins

Figure 1.5 Disease associated with specific defects in the mitochondrial respiratory chain. Nuclear-encoded proteins associated with disease are represented as white ovals. mtDNA-encoded proteins associated with disease are represented as dark gray ovals. Diseases associated with specific complexes are indicated as shown. Adapted from Schon (2000).

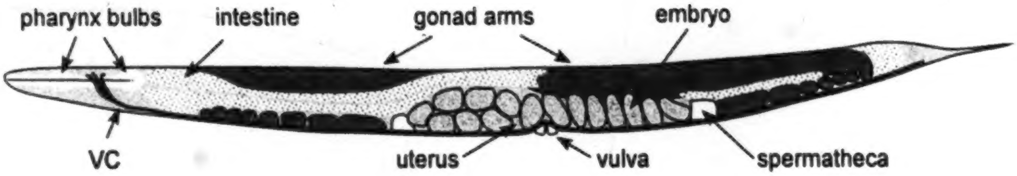
pharynx bulbs
intestine
gonad arms
embryo
VC
uterus
vulva
spermatheca

Figure 1.6 Schematic showing the main anatomical features of the *C. elegans* adult hermaphrodite.

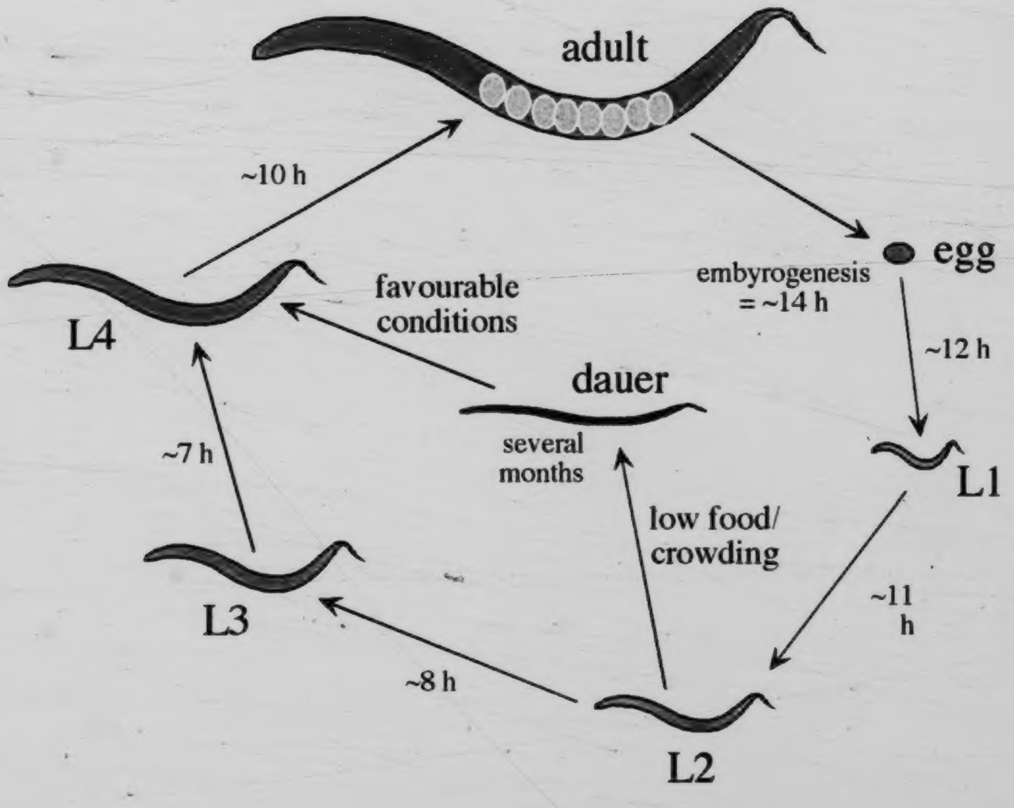
adult
~10 h
egg
embyrogenesis
= ~14 h
favourable
conditions
L4
~12 h
dauer
several
months
~7 h
L1
low food/
crowding
~11
h
L3
~8 h
L2

Figure 1.7 ***C. elegans*** **life cycle.** Times between developmental stages are based on growth at 25 °C. Adapted from Wood (1988).

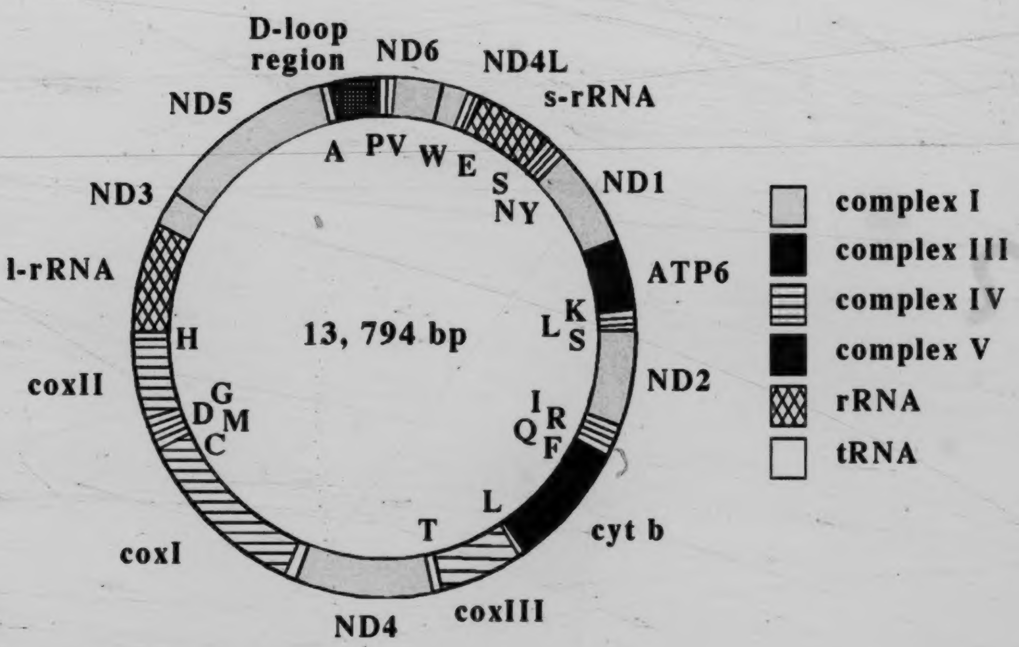
D-loop region
ND6
ND4L
s-rRNA
ND5
A
P V
W
E
S
N Y
ND1
ND3
ATP6
l-rRNA
K
L
S
13, 794 bp
H
ND2
coxII
G
D
M
C
I
R
Q
F
L
T
cyt b
coxI
ND4
coxIII
complex I
complex III
complex IV
complex V
rRNA
tRNA

Figure 1.8 Circular map of the *C. elegans* mtDNA. Each tRNA gene is identified by its one letter amino-acid code.

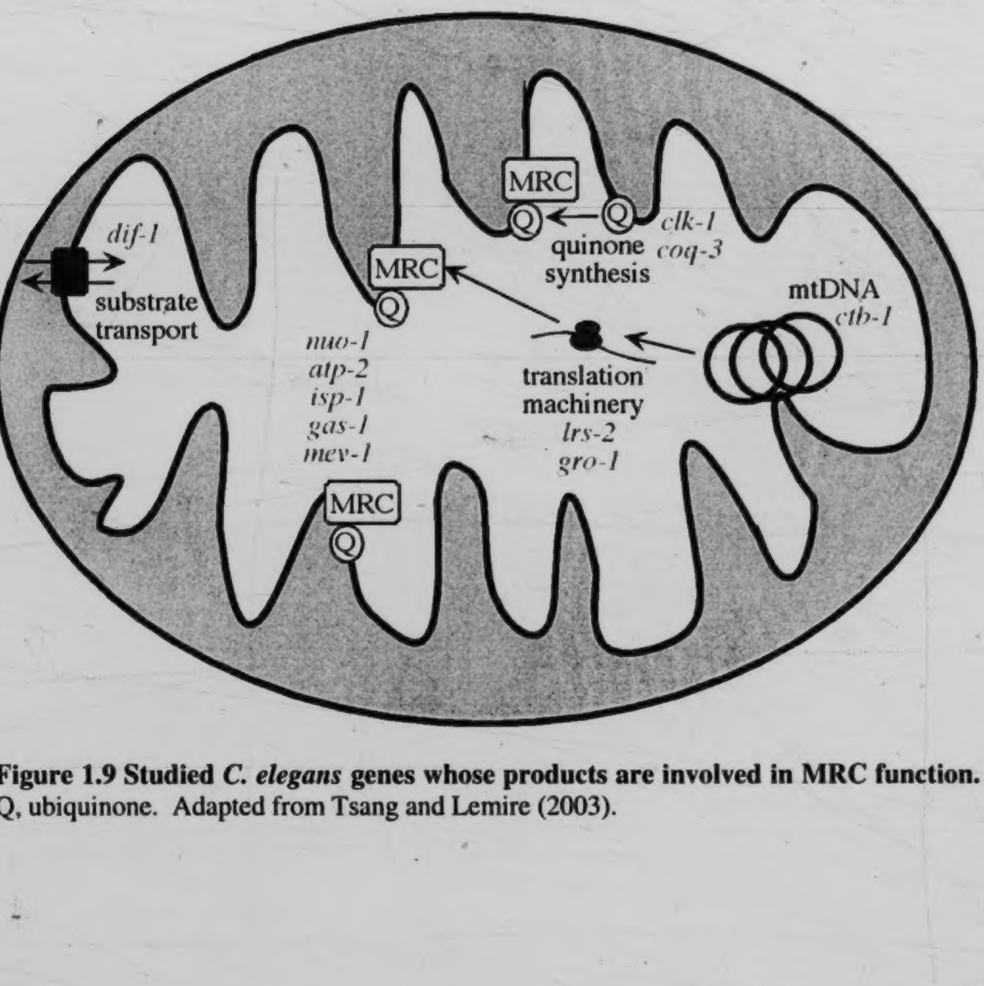
dif-1
substrate transport
MRC
Q
clk-1
coq-3
quinone synthesis
mtDNA
ctb-1
nuo-1
atp-2
isp-1
gas-1
mev-1
translation machinery
lrs-2
gro-1

Figure 1.9 Studied *C. elegans* genes whose products are involved in MRC function. Q, ubiquinone. Adapted from Tsang and Lemire (2003).

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Chapter 2

Modeling Pathogenic Human Complex I Mutations in *C. elegans*

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2.1 Introduction

The primary function of mitochondria, mediated by the mitochondrial respiratory chain (MRC), is to provide energy via oxidative phosphorylation. This energy is essential to most eukaryotic cells as it drives the majority of cellular processes. The MRC is composed of four membrane-bound electron-transporting protein complexes (I-IV) that generate a proton gradient across the mitochondrial inner membrane and the ATP synthase (complex V) that uses the proton gradient for ATP synthesis. Defects in one or more of these complexes can result in a large variety of diseases often with multisystemic presentations. Mitochondrial dysfunction occurs with an estimated incidence of 1 in 10,000 live births and is often caused by a deficiency in complex I, the NADH-ubiquinone oxidoreductase (Robinson, 1998; Triepels *et al.*, 2001). Mammalian complex I is the largest MRC complex, containing at least 46 nuclear- and mitochondrial-DNA encoded subunits, a flavin mononucleotide (FMN) cofactor, and up to eight iron-sulfur clusters (Walker, 1992; Yagi and Matsuno-Yagi, 2003; Yano, 2002). The enzyme couples electron transfer from NADH to ubiquinone with proton pumping across the mitochondrial inner membrane into the intermembrane space. Complex I dysfunction is linked to myopathies, encephalomyopathies, and neurodegenerative disorders such as Parkinson's disease and Leigh syndrome (Robinson, 1998; Shoubridge, 2001b; Smeitink *et al.*, 1998; Triepels *et al.*, 2001).

The human nuclear DNA-encoded gene *NDUFV1* encodes the 51-kDa subunit of complex I, which carries the NADH-binding site as well as FMN and iron-sulfur cluster cofactors. Several mutations in *NDUFV1* have recently been identified (Bénit *et al.*, 2001; Schuelke *et al.*, 2002; Schuelke *et al.*, 1999). Two patients reported as compound heterozygotes for the missense T423M and the nonsense R59X mutations presented with muscular hypotonia, myoclonic epilepsy, brain atrophy, and elevated lactate and pyruvate concentrations in the blood and cerebrospinal fluid. Both died before the age of 18 months (Schuelke *et al.*, 1999). A patient homozygous for the A341V mutation presented with similar symptoms, as well as macrocystic leukodystrophy (Schuelke *et al.*, 1999). At the time of the report, this patient, age ten was severely spastic and blind (Schuelke *et al.*, 1999). A more recent investigation identified six additional mutations including a deletion, an inversion, and the four single amino-acid substitutions: Y204C, C206G, E214K, and A432P (Bénit *et al.*, 2001). Patients presented with hypotonia, ataxia, psychomotor retardation, or Leigh syndrome and most died before the age of three years as a result of acute metabolic acidosis (Bénit *et al.*, 2001). Each of the six missense mutations in *NDUFV1* affect highly conserved amino acids, suggesting these may be functionally important. It seems unlikely that low cellular energy can be entirely responsible for the diversity and severity of the *NDUFV1*-related disease and of mitochondrial disorders in general, implying other molecular mechanisms are involved in pathogenesis. Unfortunately, due to the rarity of most mitochondrial disorders, these mechanisms remain poorly understood.

In this study, we have used the free-living soil nematode, *Caenorhabditis elegans*, to investigate the pathogenesis of complex I disorders. *C. elegans* offers numerous advantages for studying mitochondrial dysfunction (Lewis and Fleming, 1995; Wood, 1988). The organism has a life cycle of 3-4 days at 20 °C and features a simple anatomy with distinct tissue systems. Mitochondrial dysfunction can often present in a tissue-specific fashion, affecting those tissues with the greatest demand for MRC-generated

ATP. *C. elegans* is transparent; living animals can be readily examined by light microscopy for morphological or physiological abnormalities. The sequences of the nuclear and mitochondrial genomes have been determined (*C. elegans* Sequence Consortium, 1998; Okimoto *et al.*, 1992). Finally, the structure and bioenergetics of the nematode MRC are very similar to that of the mammalian MRC (Murfitt *et al.*, 1976). The *C. elegans* model system has garnered increasing popularity for understanding the mechanisms of human disease such as muscular dystrophy and Alzheimer disease (Aboobaker and Blaxter, 2000; Culetto and Sattelle, 2000), as well as other types of mitochondrial dysfunction (Denver *et al.*, 2000; Hihi *et al.*, 2002; Ishii *et al.*, 1998; Jonassen *et al.*, 2001).

Here, we investigate the mechanisms of pathogenesis of three *NDUFV1* missense mutations. We have previously isolated and characterized a mutation in the *C. elegans* *nuo-1* gene, the homologue of the *NDUFV1* gene (Tsang *et al.*, 2001). The *nuo-1(ua1)* allele is a large deletion that is homozygous lethal, resulting in developmental arrest at the third larval stage (Tsang *et al.*, 2001). We generated transgenic strains lacking endogenously expressed *nuo-1*, but expressing *nuo-1* carrying A352V, T434M, and A443F mutations, which correspond to the residues A341, T423, and A432 of the *NDUFV1* gene, for which disease causing mutations have been reported (Bénit *et al.*, 2001; Schuelke *et al.*, 1999). All three transgenes rescue the lethality of the *nuo-1* knockout background. The transgenic strains exhibit a number of morphological defects, including premature tissue degeneration and abnormal development of the reproductive system. The complex I mutations result in reduced brood sizes, decreased respiration, lactic acidosis, and hypersensitivity to oxidative stress. The lactic acidosis was responsive to the vitamins riboflavin and thiamine, and to sodium dichloroacetate, an activator of the pyruvate dehydrogenase complex. Surprisingly, mutants also displayed significant decreases in complex IV activity and in steady-state levels of COX I, a mitochondrial DNA-encoded subunit. Taken together, our data suggest that complex I mutations are pathogenic not only because they impair the oxidation of NADH, but also because they increase the production of reactive oxygen species and interfere with the function or assembly of other MRC complexes.

2.2 Materials and Methods

2.2.1 Strains

Worms were cultured as described (Lewis and Fleming, 1995). We used the following *C. elegans* strains: wild-type N2 Bristol; LB77, *nuo-1(ua1)/mIn1 [dpy-10(e128) mIs14]*II, *unc-119 (ed3)*III.

2.2.2 Plasmid Constructs

The *nuo-1(ua1)* mutation has been described previously (Tsang *et al.*, 2001). The A352V, T434M, and A443F mutations were constructed by oligonucleotide-directed mutagenesis using a megaprimer mutagenesis method by introducing the following codon conversions: GCC to GTC, ACT to ATG, and GCA to TTC, respectively (Sarkar and Sommer, 1990). All mutations were confirmed by sequencing. The *nuo-1* gene cassette consisted of a 5.4-kb *XhoI* genomic fragment from cosmid C09H10. The mutated *nuo-1* genes, including the promoter, were cloned at the unique *Xba*I site into pDP#MM016b, which carries an *unc-119(+)* gene (Maduro and Pilgrim, 1995; Praitis *et al.*, 2001).

2.2.3 Generation of Transgenic C. elegans

LB77 was transformed by microparticle bombardment using a BioRad Biolistic PDS-1000/HE (BioRad Laboratories, Hercules, CA) as described (Praitis *et al.*, 2001). Following bombardments, worms were allowed to recover at 20 °C. *unc-119* mutants, which are paralyzed, cannot form dauer larvae and die in the absence of food. After 7-14 days, plates were examined for dauer animals with wild-type motility. We confirmed transformation with *unc-119(+)* and the *nuo-1* transgenes by PCR analysis. Three independent transgenic lines were selected for each mutation.

2.2.4 Phenotypic Analyses

For lifespan measurements, gravid adults were allowed to lay eggs on unseeded plates for 6 h at 20 °C. Once hatched, L1 animals were transferred to separate plates and incubated at 20 °C. Animals were monitored daily and scored as dead when they no longer responded to gentle prodding on the head. Animals were transferred daily during egg laying to keep them separate from their progeny.

To determine brood sizes, individual L4 animals were transferred to separate plates, monitored daily during egg laying, and transferred to fresh plates to keep them separate from their progeny. Plates with eggs were incubated an additional 24 h to allow hatching and unhatched eggs and larvae counted. This continued until adult animals no longer laid eggs.

Newly hatched L1 animals were transferred to separate plates and incubated at 20 °C. Animals were monitored every 12 h and were scored as L4 larvae when the crescent-shaped immature vulval structure was observed. For morphological analysis, animals were mounted on 2% agarose pads and observed under a Zeiss Axioskop-2 research microscope with a SPOT-2 digital camera (Carl Zeiss Canada, Ltd., Calgary, Canada).

2.2.5 Isolation of Mitochondria

All procedures were performed at 0-4 °C. Worms were harvested from liquid culture and washed repeatedly in S Basal Complete medium to remove bacteria (Sulston and Hodgkin, 1988). Washed worms were suspended in MSEP buffer (0.2 M mannitol, 70 mM sucrose, 0.1 M EDTA, pH 7.4, 1mM phenylmethylsulfonyl fluoride) and disrupted with 0.1 mm acid-washed glass beads in a Bead-Beater homogenizer (Biospec Products, Bartlesville, OK) for three 30 sec pulses separated by 1 min cooling intervals.

Immediately following disruption by the Bead-Beater, the slurry was homogenized by ten strokes of a glass-glass homogenizer. The homogenate was twice centrifuged (1000 x *g*, 10 min) to remove cuticle and large debris. The supernatant was centrifuged (19,200 x *g*, 10 min) to pellet mitochondria. The mitochondrial pellet was resuspended in fresh MSE (0.2 M mannitol, 70 mM sucrose, 0.1 M EDTA, pH 7.4) and re-centrifuged. The final pellet of mitochondria was resuspended in 0.5 ml MSE. Protein content was determined by the BioRad Protein Assay (BioRad Laboratories, Hercules, CA) using BSA as a standard.

2.2.6 Polarographic Analyses

Oxygen consumption rates were measured using a Strathkelvin 1302 oxygen electrode with an MT200 Mitocell respiration chamber (Strathkelvin Instruments Ltd., Glasgow, Scotland). Synchronized worms at the late L4 to early adult stages were washed free of bacteria using M9 buffer (Sulston and Hodgkin, 1988). Washed worms were resuspended in 1 ml M9 buffer and triplicate aliquots placed on glass coverslips for counting. Worms were diluted to 500 worms per 50 μl volume. 500 worms were introduced into the Mitocell chamber, which was maintained at 22 °C, and oxygen consumption was measured for a minimum of 10 min. The slopes of the linear portions of the plots were used to calculate oxygen consumption rates. A minimum of three trials was performed for each strain.

Isolated mitochondria were diluted 10-fold in 0.1 M HEPES, pH 7.4 and disrupted by sonication for 2-3 min in a Branson 1200 bath sonicator (Branson Ultrasonics Corp., Danbury, CT) in an ice-water bath. Sonicated mitochondria were centrifuged (18,500 x *g*, 30 min, 4 °C) and resuspended in fresh 0.1 M HEPES, pH 7.4.

20 μg mitochondrial protein were diluted to a volume of 60 μl and introduced into the Mitocell chamber. Basal levels of oxygen consumption were recorded for 2-4 min, followed by the addition of NADH to a final concentration of 600 μM. Rotenone was added to a final concentration of 100 nM and oxygen consumption recorded for 5-10 min. Complex I-specific NADH-dependent respiration was calculated as the rate of oxygen consumption in the presence of NADH minus the rate in the presence of NADH and rotenone. A minimum of five experiments was performed for each sample.

2.2.7 Electron Transport Chain Assays

Enzymatic activities of individual or pairs of MRC complexes were performed on an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England) essentially as described (Birch-Machin and Turnbull, 2001) using 25-50 μg of disrupted mitochondrial protein. Rotenone-sensitive NADH-decylubiquinone oxidoreductase activities were measured at 340 nm using 65 μM 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone as electron acceptor, 2 μg/ml antimycin A, and 0.13 mM NADH. Rotenone was added to 2 μg/ml. Malonate-sensitive succinate-dichlorophenolindophenol reductase activity was measured at 600 nm after preincubating mitochondria in assay medium (2.5 mM KH_2PO_4, 0.05 mM EDTA, 10 mM succinate, 1.6 mM KCN, 1 mg/ml BSA, pH 7.4) in the presence of 2 μg/ml rotenone and 5 μg/ml antimycin A for 10 min at 30 °C. Reactions were started by the addition of 50 μM decylubiquinone and 75 μM dichlorophenolindophenol. Malonate was added to 25 mM. Antimycin A-sensitive decylubiquinol-cytochrome *c* reductase activity was measured at 550 nm after preincubating mitochondria in assay medium (25 mM KH_2PO_4, 5 mM $MgCl_2$, 2.5 mg/ml BSA, 2 mM KCN, pH 7.4) in the presence of 50 μM cytochrome *c* (III), and 2 μg/ml rotenone for 10 min at 30 °C. Assays were started by adding 80 µM decylubiquinol. Antimycin A was added to 5 µg/ml. Succinate-cytochrome *c* reductase activity was measured at 550 nm after preincubating mitochondria in assay medium (0.1 M Tris-SO_4, pH 7.4, 2 mM KCN, 1 mg/ml BSA) in the presence of 10 mM succinate and 2 µg/ml rotenone for 10 min at 30°C. Assays were started by the addition of 50 µM cytochrome *c* (III). Antimycin A was added to 5 µg/ml. Glycerol-phosphate-cytochrome *c* reductase was measured as for succinate-cytochrome *c* reductase activity, except 20 mM glycerol-1-phosphate was added instead of 10 mM succinate. Cytochrome *c* oxidase activity was measured at 550 nm using 15 µM cytochrome *c* (II) as electron donor in the presence of 0.45 mM lauryl maltoside. Potassium cyanide was added to 1 mM.

2.2.8 Supplementation Assays

Individual L4-staged transgenic animals were transferred to plates supplemented with 5 µg/ml sodium dichloroacetate, 0.1 mM ascorbate, 1 µg/ml thiamine, 1 µg/ml riboflavin, 1 mg/ml oxaloacetate, or without supplement and incubated for 7 days at 20 °C in the dark. These concentrations of supplements were chosen as optimal after a series of smaller scale trial experiments. Animals were monitored daily during egg laying and transferred to fresh plates to keep them separate from their progeny. Only hatched progeny were scored. A minimum of 24 broods was counted for each strain and condition.

2.2.9 Measurements of Lactate and Pyruvate Concentrations

Worms cultured in liquid medium with or without supplementation were harvested and aliquots counted to determine approximate worm concentrations of cultures. ~1.0×10^7 animals were washed repeatedly to remove bacteria and resuspended in a final volume of 30 ml of 5% trichloroacetic acid. Worms were sonicated on ice with four 30-sec pulses interspersed with 30-sec cooling periods using a Branson Sonifier 450 (Branson Ultrasonics Corp,, Danbury, CT) set to a power level of 450 W. The lysate was centrifuged (10,000 x *g*, 10 min, 4 °C) to pellet precipitate and debris. The supernatant was neutralized with 5 N KOH and clarified with a second centrifugation (10,000 x *g*, 10 min, 4 °C). Lactate and pyruvate concentrations were measured using Sigma diagnostics kits (Sigma Diagnostics, St. Louis, MO).

2.2.10 Cytochrome c Oxidase Histochemistry and TUNEL Labeling

Cuticles were permeabilized for histochemical staining as described (Xie *et al.*, 1995). The cytochrome *c* oxidase staining protocol was adapted for *C. elegans* from (Sciacco and Bonilla, 1996). Briefly, synchronized animals with permeabilized cuticles were incubated in 1 ml phosphate buffer (5 mM potassium phosphate, pH 7.4) containing 0.1% 3,3'-diaminobenzidine, 0.1% cytochrome *c*, and 0.02% catalase, for 75 min at 37 °C in the dark with constant rotation. Control reactions were incubated in the presence of 10 mM KCN to inhibit complex IV activity. Stained worms were centrifuged for 10 sec (360 x *g*) and the supernatant was removed. The worm pellet was washed three times for 5 min in phosphate buffer. Stained animals were mounted and photographed.

For detection of apoptotic nuclei based on TUNEL, synchronized young adult hermaphrodites with permeabilized cuticles were labeled using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN) according to product instructions. Stained animals were mounted and photographed using fluorescence optics.

2.2.11 Electrophoresis and Western Blot Analyses

Known numbers of worms were washed, lysed by boiling in loading buffer, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%

acrylamide) (Tsang *et al.*, 2001). Proteins were transferred electrophoretically to nylon membranes. Blots were treated with mouse monoclonal antiserum against the human COXI protein (Molecular Probes, Eugene, OR). Detection was with a peroxidase-labeled goat anti-mouse secondary antibody and the Enhanced Chemiluminescence Western Blotting System (Amersham Biosciences, Buckinghamshire, UK). Blots were stripped and re-probed with rabbit polyclonal antiserum raised against *Saccharomyces cerevisiae* Atp2p (Tsang *et al.*, 2001). The relative amounts of protein were quantified from films using the BioRad Gel Doc 1000 image analysis system and Molecular Analyst software (BioRad Laboratories, Hercules, CA).

2.3 Results

2.3.1 Generation of Transgenic C. elegans Strains Expressing nuo-1 Mutations

The *C. elegans* NUO-1 protein shares 75% amino acid sequence identity with the NDUFV1 protein (Figure 2.1). Mutated versions of the *nuo-1* gene carrying the A352V, T434M, or A443F mutations were generated (Figure 2.2). These three amino acids are conserved in the NDUFV1 protein and correspond to A341, T423, and A432, respectively. The A352V and T434M mutations exactly mimic the disease-causing *NDUFV1* A341V and T423M mutations. The *nuo-1* A443F mutation, previously created in our lab, is similar to the *NDUFV1* A432P mutation. Each mutated *nuo-1* gene was transformed with an *unc-119(+)* co-transformation marker into LB77 (*nuo-1(ua1)*/+) via microparticle bombardment (Praitis *et al.*, 2001). LB77 is also homozygous for the *unc-119(ed3)* mutation, which renders the animals partially paralyzed and unable to form dauer larvae, an alternate life stage that allows *C. elegans* to survive for months without feeding. Successful transformants regain mobility and the ability to enter the dauer state. Transformation by microparticle bombardment produces extrachromosomal arrays of variable mitotic and meiotic stability (Mello and Fire, 1995) or, at a lower frequency, the DNA can be integrated into the genome (Praitis *et al.*, 2001). Three independent transformants were selected by complementation of the *unc-119* defects for each mutation. Homozygous *nuo-1(ua1)* progeny carrying the *nuo-1* transgenes were subsequently isolated. The genotypes of all transgenic lines were confirmed by PCR analysis. For all three mutations, each independent line gave similar results and one was chosen for more detailed examination. The *nuo-1* A443F and A352V transgenes were present as extrachromosomal arrays with transmission rates of 67 ± 27% and 60 ± 19%, respectively. The T434M transgene was integrated and maintained in the homozygous state.

2.3.2 Characterization of Transgenic Strains

Each of the *nuo-1* transgenes complemented the lethality of the *nuo-1(ua1)* background and supported full development to reproductive adults. We conclude that the mutant NUO-1 proteins are expressed and functionally assembled into complex I, which was sufficiently active to support the energy generation needed for development. Progeny of the A443F and A352V transgenic lines that had lost the extrachromosomal arrays arrested as embryos, a phenotype similar to that seen with *nuo-1* RNA interference (Tsang *et al.*, 2001).

Growth, reproduction, and aging are biological processes that depend on the integrity and function of the MRC (Tsang *et al.*, 2001; Vanfleteren and De Vreese, 1996). We observed that the three transgenic mutants had variably decreased reproductive capabilities as measured by their brood sizes; the A443F mutation has the most severe effect (Figure 2.3A). All three mutant strains have significant decreases in lifespan; again, the A443F mutation has the most deleterious effect (Figure 2.3B). Because *C. elegans* development relies heavily on MRC function, we examined the rates of larval development for the *nuo-1* transgenic strains. We measured the time needed for animals to develop from newly hatched L1 larvae to the L4 larval stage, which is easily recognized by the presence of the crescent-shaped immature vulva. In wild type animals, approximately 46 h are needed. A significant developmental lag of approximately 12 h was observed with A352V mutant animals (Figure 2.3C). Thus, the mutated *nuo-1* genes are hypomorphic alleles, which are sufficiently functional to rescue the lethality of the null *nuo-1(ua1)* allele but do not confer wild type fertility, lifespan, or development.

2.3.3 Gonadal Abnormalities and Premature Aging in Transgenic Mutants

We observed the first signs of impaired gonadal development when the reproductive system is rapidly maturing during the L4 and early adult stages. Normally, by the late L4 stage, the arms of the gonad are fully extended anteriorly and posteriorly along the ventral surface and reflexed along the dorsal surface, forming a U-shaped structure (Schedl, 1997). The distal portion of each gonad arm contains syncytial germline nuclei incompletely surrounded by membranes (Figure 2.4A). As *C. elegans* matures into a reproductive adult, membranes surround individual nuclei as they migrate towards the proximal ends of the gonad, forming oocytes that are fertilized while passing through the spermatheca. Fertilized oocytes are then expelled from the uterus through the vulva. Somatic gonad development was severely affected in transgenic animals expressing the A443F and A352V mutations and moderately affected in the T434M mutant. In early adult animals, gonad arms were usually shorter than normal, suggesting difficulties with distal tip cell migration, and appeared shriveled, leaving space between the gonad and the body wall (Figure 2.4B-D). Furthermore, the syncytial germline nuclei were difficult to identify and more disorganized than in wild-type gonads. As the transgenic animals matured into gravid adults, oocyte formation proceeded but more slowly than normal, suggesting that meiotic germline division or oogenesis are compromised by mitochondrial dysfunction (Figure 2.4F-H). Vulval development was slower in more severely affected mutant animals but otherwise appeared complete. The decreases in brood sizes are not due to an egg-laying defect but rather are the result of impaired germline development.

MRC function is closely connected to lifespan determination and aging (Tsang and Lemire, 2003). Both germline and somatic tissue deterioration increases significantly with age (Garigan *et al.*, 2002). The overall health of *nuo-1* transgenic animals declined rapidly once they had reached reproductive maturity. The transgenic mutants accumulated a dark pigment, a biomarker of aging, within two days of becoming gravid adults; pigment accumulation occurs in wild-type *C. elegans* towards the end of their reproductive period, which is approximately 4 days into adulthood (Garigan *et al.*, 2002). Microscopic analysis revealed prominent vacuolar structures that appeared throughout the body, usually beneath the cuticle, suggesting the degeneration of body wall muscle tissue (Garigan *et al.*, 2002; Herndon *et al.*, 2002). The vacuolar structures in the transgenic mutants became apparent on the first day of adulthood (Figure 2.5B-D) about 4 days earlier than in wild-type animals (Figure 2.5A, E) and accumulated as the animals aged (Figure 2.5F-H). The A352V and A443F mutants are the most severely affected, while the T434M mutant displayed a milder phenotype.

Apoptosis and mitochondrial dysfunction are intimately related (Chan, 2005; Kirkland and Franklin, 2003). We further examined the severe mutants A352V and A443F for the presence of premature apoptosis in young adults. Both transgenic mutants showed the accumulation of punctate TUNEL-specific staining throughout the body (Figure 2.6C, D), suggesting the presence of apoptotic-induced nuclear DNA fragmentation. TUNEL-positive cells were not identified in identically treated young adult N2 nematodes (Figure 2.6A), but only observed when treated with DNase I, which induces DNA strand breaks, serving as a positive control for TUNEL labeling (Figure 2.6B).

2.3.4 Impaired Respiration and Lactic Acidosis in Transgenic Mutants

We investigated the effects of the three complex I mutations on respiration by monitoring oxygen consumption of live animals. Respiration values were normalized to rates per 1,000 animals or to protein content. The percentages of respiratory activity in the transgenic lines as compared to the wild type are very similar for both methods of calculation because the sizes of transgenic and wild type animals are not significantly different (not shown). The A352V and A443F mutants consumed oxygen at rates approximately half the wild-type rate, while the T434M mutant had a rate ~65% of the wild-type rate (Table 2.1). These rates correlate well with the severity of the mutations as measured by brood sizes or lifespan. We also determined the rotenone-sensitive NADH-oxidase activities of isolated mitochondria. The mitochondria were disrupted and a membrane fraction isolated to allow access of NADH to the matrix-facing NADH-binding site of complex I. Rotenone is a specific inhibitor for complex I and rotenone-sensitive respiration is attributable to complex I activity. In agreement with the live animal respiration rates, the A443F and A352V mutations had the most severely affected rotenone-sensitive respiration rates (23% and 37% of wild-type, respectively; Table 2.2). Complex I-mediated respiration in the T434M transgenic strain was reduced to 52% of wild-type. The relative oxygen consumption rates of live animals (Table 2.1) are higher than the complex I-specific rates (Table 2.2), possibly indicating the preferential use of other respiratory substrates over NADH in live animals.

Impaired complex I-dependent respiration will lead to excess cellular NADH, favoring the reduction of pyruvate to lactate. We found that pyruvate concentrations were decreased and lactate concentrations were increased in the mutants compared to the wild-type, resulting in greatly elevated lactate/pyruvate ratios (Table 2.3). Again, the A443F mutant demonstrated the largest change with a ratio 6.3-fold higher than the wild-type. These data suggest that the *nuo-1* mutations may indirectly impede the citric acid cycle and oxidative phosphorylation by depleting the cellular pools of pyruvate. In addition, the pyruvate dehydrogenase complex is inhibited by phosphorylation as a result of the activation of the pyruvate dehydrogenase kinase by NADH (Chen *et al.*, 1999). Thus, the increased levels of NADH shifts equilibrium towards the reduction of pyruvate to lactate by lactate dehydrogenase and result in lactic acidosis.

2.3.5 Pharmacological Treatment of Transgenic Mutants

A variety of pharmacological agents, including vitamins, have been used to treat mitochondrial diseases (Chinnery and Turnbull, 2001; Tarnopolsky and Beal, 2001; Taylor *et al.*, 1997). We picked L4-staged transgenic animals to individual plates supplemented with sodium dichloroacetate, thiamine, and riboflavin, activators of the pyruvate dehydrogenase complex that are used clinically to treat lactic acidosis (Chinnery and Turnbull, 2001; Tarnopolsky and Beal, 2001; Taylor *et al.*, 1997) and allowed them to lay their broods. Riboflavin, as a precursor of FMN, is also essential for complex I function. We also investigated the effects of ascorbate, a free-radical scavenger, and oxaloacetate, an intermediate of the citric acid cycle. All three mutant strains, but not the wild-type, showed significant increases in brood size when supplemented with 1 μg/ml riboflavin (Figure 2.7). Significant increases in brood size were also observed for the

A352V and A443F mutants when supplemented with 5 μg/ml sodium dichloroacetate or 0.1 mM ascorbate. Thiamine supplementation (1 μg/ml) significantly increased the brood size of the A352V mutant. None of the mutants benefited significantly from 1 mg/ml oxaloacetate. The reproductive fitness of all three *nuo-1* mutant strains can be improved by pharmacological intervention. Each mutant strain benefits from a specific set of supplements, suggesting that the metabolic consequences of each mutation may not be identical in each of the three transgenic strains.

To investigate further the mechanisms by which the supplements exert their effects, we examined the lactate/pyruvate ratios of sodium dichloroacetate or riboflavin-treated A352V and A443F mutants. These two supplements were chosen because they consistently showed the greatest benefit. Lactate concentrations were significantly decreased and pyruvate concentrations significantly increased by sodium dichloroacetate, resulting in markedly lower lactate/pyruvate ratios for both mutants (Table 2.4). Supplementation with riboflavin showed similar results for the A443F mutant, but had no significant effect on the A352V mutant strain (Table 2.4). These results suggest that lactic acidosis is responsible for at least some of the pathogenic consequences of all three *nuo-1* mutations. Treatments that ameliorate the acidosis can markedly decrease the severity of the phenotype. Interestingly, the beneficial effects of riboflavin on the brood sizes of the A352V mutant (Figure 2.7) do not appear to be associated with a reduction in lactate/pyruvate ratios (Table 2.4). We suggest that riboflavin improves A352V mutant metabolism in a manner that is mechanistically different than sodium dichloroacetate.

2.3.6 Complex IV Deficiency in Mutant nuo-1 Transgenic Strains

Impaired metabolism caused by a defect in one MRC complex is often associated with altered levels of the other MRC complexes (Heddi *et al.*, 1999; Kayser *et al.*, 2001; Murdock *et al.*, 1999; Wiesner *et al.*, 1999). We investigated the levels of cytochrome *c* oxidase activity in mutant and wild-type animals by histochemical staining; the complex IV-dependent oxidation of diaminobenzidine produces a brown pigment. Cytochrome *c* oxidase activity was diminished in the body wall and pharyngeal muscles of L3 larvae from all three transgenic mutants (Figure 2.8A). The A352V and A443F mutants were more severely affected than the T434M mutant. As the mutants matured, cytochrome *c* oxidase activity increased, but failed to reach wild-type levels (Figure 2.8B).

To determine whether the observed decreases in cytochrome *c* oxidase activity are due to diminished levels of assembled complex IV, we performed Western blot analyses with an antiserum directed against human COXI, a mitochondrial DNA-encoded subunit of the complex IV catalytic core. COXI levels were consistently lower in the *nuo-1* mutant strains than in the wild-type (Figure 2.8C). When normalized to ATP-2 levels, a subunit of the ATP synthase, the A443F, A352V, and T434M mutants had COXI levels of 43±12%, 51±17%, and 90±3% of the wild-type, respectively.

To further assess the effects of the complex I mutations on the MRC, we measured the enzymatic activities of the individual or pairs of MRC complexes in wild type and transgenic mitochondrial membranes (Table 2.5). As expected, the rotenone-sensitive NADH-decylubiquinone reductase activities in all three mutants were significantly reduced. The cyanide-sensitive cytochrome *c* oxidase activities were also severely affected. The complex II and complex III activities were either modestly affected or unchanged with respect to the wild type. Several lines of evidence (histochemical staining (Figure 2.8A, B), respiration measurements (Table 2.2), Western blots (Figure 2.8C), and activity measurements (Table 2.5)) indicate that the complex I mutations impair the assembly of functional complex IV.

2.3.7 nuo-1 Mutants are Hypersensitive to Oxidative Stress

Reactive oxygen species are generated as a normal by-product of mitochondrial respiration and can damage cellular proteins, lipids, and DNA (Raha and Robinson, 2001). Mitochondrial dysfunction can result in accelerated rates of reactive oxygen species generation (Senoo-Matsuda *et al.*, 2001). We subjected the *nuo-1* mutants to two forms of oxidative stress, hyperoxia and the herbicide, paraquat. Paraquat is believed to increase the production of superoxide, although its site of action is not known. All three mutants are hypersensitive to hyperoxia, with the A352V mutant being particularly so (Figure 2.9A). Similarly, mutant animals were significantly more sensitive to 0.2 mM paraquat than the wild-type (Figure 2.9B). The sensitivity of the A352V mutant but not the T434M or the A443F mutants to hyperoxia could be significantly reduced by supplementation with either sodium dichloroacetate or riboflavin (Figure 2.9C). Sodium dichloroacetate increased the survival of all three mutants challenged with paraquat (Figure 2.9D), while riboflavin improved survival of the A352V and the A443F mutants.

2.4 Discussion

For most mitochondrial diseases, the molecular mechanisms of pathogenesis are only poorly understood, in part because of the rarity of these diseases, which limits the availability of materials for detailed investigations. In this study, we mimicked pathogenic human mutations of the *NDUFV1* gene by using the *C. elegans* homologue, *nuo-1*. The *nuo-1* A352V, T434M, and A443F mutations complement the lethality of the *nuo-1* deletion background, resulting in viable strains that could be analyzed in detail. The ability to tolerate mutations that would otherwise be lethal in more complex organisms may be an additional advantage of the nematode model system.

Reproduction and development in *C. elegans* are energy-intensive and rely heavily on MRC function (Tsang and Lemire, 2002; Tsang and Lemire, 2003; Tsang *et al.*, 2001; Vanfleteren and De Vreese, 1996). All three mutants suffered decreased brood sizes, with the A443F mutation having the most severe effects and the T434M mutation having the mildest. The A352V mutant exhibited a significant developmental lag during larval development. Morphological examination of the mutants revealed somatic gonad arms that had failed to fully extend and disorganization of the germline nuclei. Oogenesis requires the synthesis of large numbers of copies of mitochondrial DNA as well as the storage of maternally-derived products needed to support embryonic and early larval development (Piano *et al.*, 2000; Tsang and Lemire, 2002; Tsang *et al.*, 2001). It is not surprising that a normally functioning MRC is needed for reproductive fitness.

A defect in complex I may create three problems: 1) the MRC will be unable to oxidize NADH and the redox balance of the cell will be perturbed; 2) complex I-mediated proton pumping may be impaired leading to a decreased ability to synthesize ATP; 3) oxygen free radical production may be accelerated (Seo *et al.*, 1998).

Our data strongly support a role for oxidative damage in the pathogenesis of the *nuo-1* mutations. Complex I is a major site of oxygen radical production in the MRC (Raha and Robinson, 2000; Raha and Robinson, 2001). The oxidation of reduced flavin by molecular oxygen can produce superoxide or peroxide products (Imlay, 2003). The NUO-1 subunit harbors an FMN cofactor and the mutations we introduced may increase access of oxygen to the reduced flavin. All three mutants are hypersensitive to the oxidative stresses, hyperoxia and paraquat, with the A352V mutant being the most affected. We believe the mutations increase the endogenous production of oxygen free radicals making the cell's defense mechanisms less able to deal with the additional stresses. The beneficial effects of supplementation with ascorbate, an antioxidant, on the reproductive capabilities of the A352V and A443F mutants also suggest that oxidative stress contributes to the pathogenesis of these mutations. All three transgenic mutants suffered shortened lifespans and exhibited signs of premature aging; vacuolar structures that appear to be associated with the degeneration of the body wall muscle became apparent in one day-old mutant adults. These structures are only found in wild-type adults near the end of their egg laying period, roughly four days into adulthood. Along with the early accumulation of dark pigment in the mutants, the vacuolar structures and presence of TUNEL-positive cells serve as biomarkers of premature senescence and are particularly prominent in the A352V and A443F mutants. Premature aging and oxidative damage are closely linked in *C. elegans* (Ishii *et al.*, 1998; Ishii *et al.*, 2002; Melov *et al.*, 2000; Senoo-Matsuda *et al.*, 2001) and other organisms (Melov, 2002). It is unclear if the oxidative damage, premature apoptosis, and vacuolar structures observed in transgenic mutants are part of the same pathogenic pathway, or occur independently in parallel. Determining the exact site of oxygen radical production in the MRC and the direct trigger of apoptosis in the mutants will further increase our understanding of the molecular mechanisms of pathogenesis.

Inefficient oxidation of cellular NADH also contributes to pathogenesis. Lactic acidosis resulting from impaired utilization of pyruvate by the citric acid cycle, increased glycogen utilization, and increased glycolytic activity, is a hallmark of complex I dysfunction (Loeffen *et al.*, 2000) and of mitochondrial disease in general (DiMauro *et al.*, 1985). The three-dimensional structure of the NUO-1 protein is not known, but the probable locations of the NADH, FMN, and iron-sulfur cluster binding sites in this protein have been suggested (Walker, 1992). None of the three mutations we studied are located within these sites (Figure 2.2), making it difficult to assign them a probable mechanism of action. The mutations may destabilize the 51-kDa subunit, impede its ability to assemble into the complex, or they may affect catalysis by impairing substrate binding or electron flow. The lack of antibodies against subunits of complex I has hampered our investigation of complex I assembly. Whatever the mechanism of action of the mutations, the oxygen consumption data indicate that the complex I mutations significantly reduce the rates of NADH-dependent respiration via the MRC. Consequently, the *nuo-1* mutants have significantly higher lactate concentrations and lactate/pyruvate ratios (3 to 6-fold; Table 2.3). Significant improvements in reproduction of all three transgenic mutants are seen in the presence of sodium dichloroacetate, riboflavin, or thiamine. These three supplements are activators of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-coenzyme A for further metabolism in the citric acid cycle. In the presence of high levels of NADH, the pyruvate dehydrogenase complex is inactivated by the NADH-stimulated phosphorylation of the pyruvate dehydrogenase complex by the pyruvate dehydrogenase kinase (Chen *et al.*, 1999; Tarnopolsky and Beal, 2001). Sodium dichloroacetate inhibits the kinase. Riboflavin and thiamine are vitamin precursors for pyruvate dehydrogenase complex cofactors. Each of the supplements may stimulate the complex and lead to an increase in substrate for the citric acid cycle and a decrease in pyruvate available for reduction to lactate. Riboflavin, as a precursor to FMN, may also benefit the three mutants by directly improving complex I function. That riboflavin supplementation benefits the A352V mutant (Figure 2.7) without significantly reducing its lactate/pyruvate ratio (Table 2.4) may be indicative of such a mechanism, but a more direct investigation of complex I catalytic properties will be needed to confirm this.

The inefficient oxidation of cellular NADH may also contribute to the hypersensitivity of the mutants to hyperoxia and paraquat. The more reducing cellular environment of the mutants is likely to facilitate the inappropriate reduction of oxygen by reduced electron carriers. The beneficial effects of sodium dichloroacetate and riboflavin on mutant survival under oxidative stress may result from the ability of these supplements to stimulate NADH oxidation and produce a less reducing cellular environment (Figure 2.9C, D).

Surprisingly, the *nuo-1* mutations result in a complex IV deficiency as judged by decreased cytochrome *c* oxidase activities (Table 2.5) and by lower steady-state levels of COXI subunit (Figure 2.8C). How can missense mutations in complex I affect complex

IV activity? In yeast mutants that affect the assembly of complex IV, lower levels of the mitochondrial DNA-encoded COX subunits COXI, II, and III, are often observed (Glerum *et al.*, 1995; Shoubridge, 2001a). In mammalian mitochondria, the majority of complex I is physically associated with complex III and a minority is present in a supercomplex of complexes I, III, and IV (Schägger and Pfeiffer, 2000). Supercomplexes have been postulated to enhance substrate channeling, catalysis, and the sequestration of reactive intermediates that may lead to the production of oxygen free radicals (Schägger and Pfeiffer, 2000). Complex III levels in the mutants are not severely affected. This argues against a role for supercomplex formation in the complex IV decreases we observed. Although we do not understand how, we believe that this is the first demonstration of defined complex I mutations causing a complex IV assembly defect in a model organism.

In conclusion, we have shown that human disease-causing mutations in the 51-kDa subunit of complex I can be successfully modeled in *C. elegans*. The nematode mutants displayed features that parallel those elicited by *NDUFV1* mutations such as increased lactate/pyruvate ratios and decreased NADH-dependent mitochondrial respiration. In addition, the *C. elegans* mutants revealed aberrant tissue degeneration and allowed us to explore the molecular mechanisms of pathogenesis of these complex I mutations through supplementation experiments. Finally, we report a previously unrecognized connection between complex I integrity and complex IV assembly.

Table 2.1 Transgenic mutants have decreased rates of oxygen consumption.

Strain	Oxygen consumption rate nmol O_2/min/1000 animals[a]	Oxygen consumption rate nmol O_2/min/mg protein[a]
N2	1.3 ± 0.12 (100%)	8.6 ± 1.1 (100%)
A352V	0.66 ± 0.11 (51%)*	4.6 ± 0.9 (54%)*
T434M	0.86 ± 0.01 (66%)*	5.4 ± 0.4 (64%)*
A443F	0.62 ± 0.02 (48%)*	4.3 ± 0.4 (51%)*

[a] Values are the means ± SD of a minimum of five trials. * $P<0.05$ compared to the N2 wild-type strain using two-sample *t*-test.

Table 2.2 Transgenic mutants have decreased rotenone-sensitive NADH-dependent oxygen consumption rates.

Strain	Oxygen consumption rate[a]
N2	187 ± 45 (100%)
A352V	70 ± 20 (37%)*
T434M	97 ± 21 (52%)*
A443F	43 ± 24 (23%)*

[a]Values are expressed as nmol O_2 min^{-1} mg $protein^{-1}$ and represent the means ± SD of a minimum of five trials. *$P<0.02$ compared to the N2 wild-type strain using a two-sample *t*-test.

Table 2.3 Transgenic mutants suffer from lactic acidosis.

Strain	[Lactate[a]]	[Pyruvate[a]]	Lactate/Pyruvate
N2	1.3 ± 0.31	0.103 ± 0.005	12 ± 3.1
A352V	3.6 ± 0.77	0.090 ± 0.008	40 ± 9.3**
T434M	2.5 ± 0.76	0.061 ± 0.008	40 ± 14*
A443F	4.4 ± 1.7	0.057 ± 0.011	77 ± 34**

[a] Values are expressed in mM and represent the means ± SD of a minimum of four trials.
*$P<0.05$, **$P<0.01$ compared to the wild-type strain using a two-sample *t*-test.

Table 2.4 Supplementation can decrease lactate/pyruvate ratios.

Strain	Supplement	[Lactate[a]]	[Pyruvate[a]]	Lactate/Pyruvate
A352V	none	3.6 ± 0.77	0.090 ± 0.008	40 ± 9.3
A352V	NaDCA[b]	3.01 ± 0.05	0.138 ± 0.004	21.8 ± 0.7**
A352V	riboflavin	3.16 ± 0.03	0.089 ± 0.009	36 ± 3.6
A443F	none	4.4 ± 1.7	0.057 ± 0.011	77 ± 34
A443F	NaDCA	1.92 ± 0.04	0.091 ± 0.005	21 ± 1.9**
A443F	riboflavin	2.86 ± 0.08	0.123 ± 0.018	23 ± 3.5**

[a] Values are expressed in mM and represent the means ± SD of a minimum of four trials. [b] NaDCA, sodium dichloroacetate. **P<0.01 compared to the unsupplemented sample using a two-sample *t*-test.

Table 2.5 Electron transport chain assays.

Assay [a]	N2	A352V	T434M	A443F
I[b]	81 ± 5	24 ± 4 ** (30%)[c]	36 ± 3** (44%)	26 ± 4** (32%)
II[d]	132 ± 9	97 ± 7*(73%)	119 ± 5 (90%)	101 ± 9*(77%)
III[e]	58 ± 4	46 ± 2 (79%)	71 ± 5* (122%)	44 ± 4* (76%)
II+III[f]	54 ± 4	43 ± 3 (80%)	76 ± 3* (141%)	35 ± 4* (65%)
G1P+III[g]	24 ± 1	19 ± 1 (79%)	25 ± 2 (104%)	11 ± 2** (46%)
IV[h]	58 ± 4	27 ± 3** (47%)	44 ± 3* (76%)	33 ± 3** (57%)

[a]Enzyme activities (nmol substrate min^{-1} mg^{-1} protein) are means ± SD. A minimum of five trials were performed for each assay and strain. [b]Complex I function was measured as the rotenone-sensitive NADH-decylubiquinone reductase activity. [c]Numbers in parentheses represent percent activity with respect to the wild-type (N2) value. [d]Complex II function was measured as the malonate-sensitive succinate-dichlorophenolindophenol reductase activity. [e]Complex III function was measured as the antimycin A-sensitive decylubiquinol-cytochrome *c* reductase activity. [f]The combined function of complexes II and III were measured as the antimycin A-sensitive succinate-cytochrome *c* reductase activity. [g]The combined function of the glycerol-phosphate dehydrogenase and complex III was measured as the antimycin A-sensitive glycerol-1-phosphate-cytochrome *c* reductase activity. [h]Complex IV function was measured as the cyanide-sensitive cytochrome *c* oxidase activity. $^{*}P<0.01$, $^{**}P<0.001$ compared to N2 wild-type activity using two-sample *t*-test.

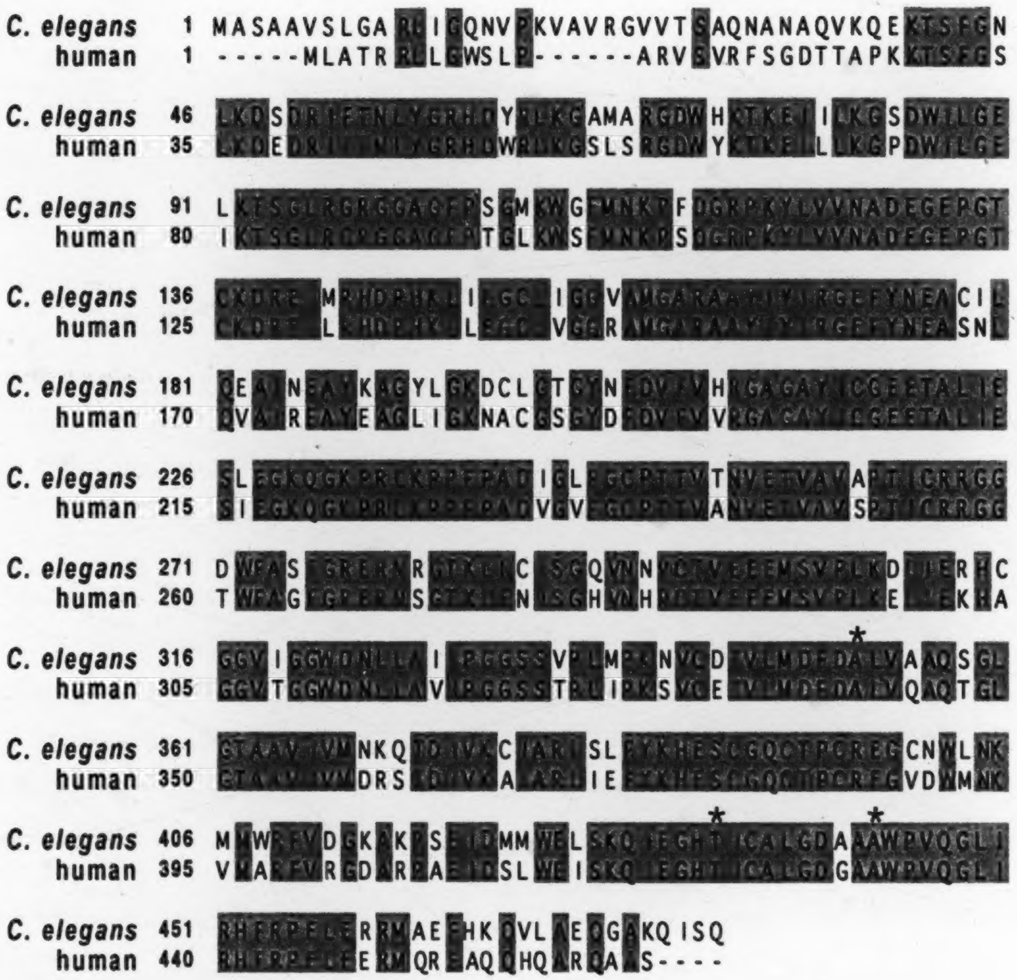


Figure 2.1 Alignment of *C. elegans nuo-1* and human *NDUFV1* protein sequences. Amino acids represented by their single letter code. Asterisks above sequence indicate amino acids involved in this study.

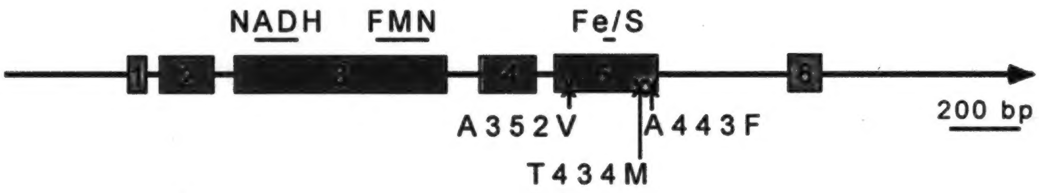

NADH
FMN
Fe/S
1
2
3
4
5
6
A352V
A443F
T434M
200 bp

Figure 2.2 Gene structure of *nuo-1*. Grey boxes indicate exon regions. Predicted substrate and cofactor binding sites (lines above exons) and missense mutations created in this study (X) are indicated. Amino-acids are referred to by their single letter code. Fe/S, iron-sulfur cluster.

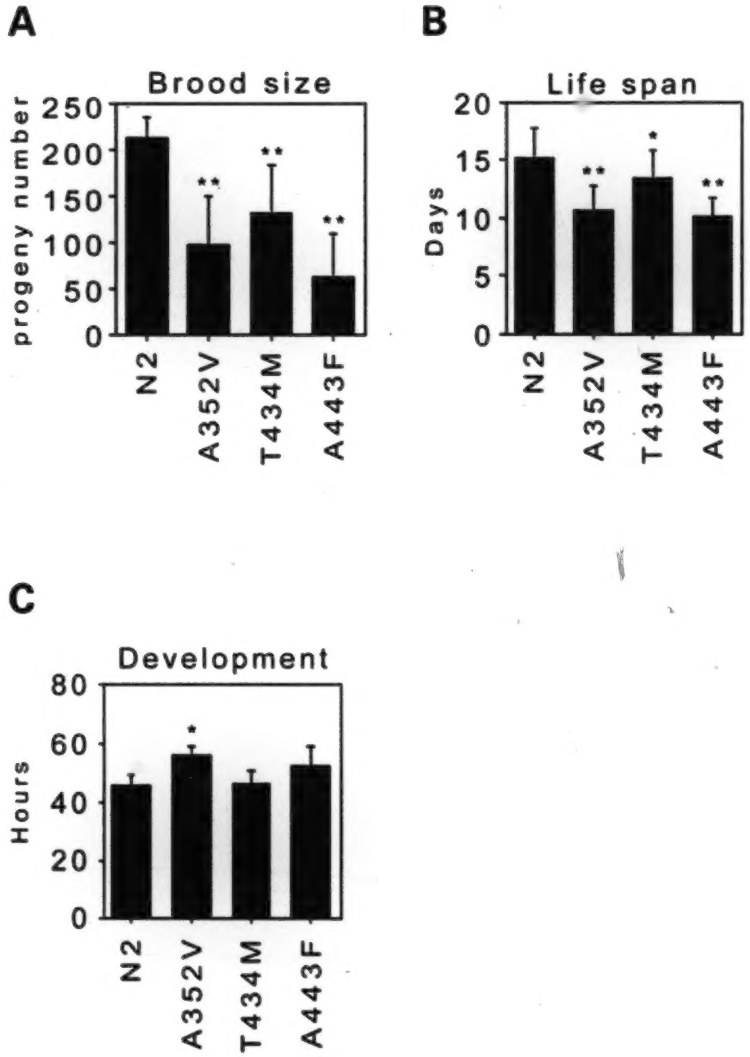


Figure 2.3 Phenotypic characterization of transgenic mutant strains. (A) Brood size measurements. Transgenic animals were allowed to lay their eggs at 20 °C and hatched and unhatched progeny were counted. Values are the average of 24 broods counted. **(B)** Life-span measurements. Animals were monitored daily and scored as dead when they no longer responded to gentle prodding on the head. Measurements were conducted at 20 °C. Values are the averages of 24 animals. **(C)** Developmental time to the L4 stage. Animals were monitored every 12 h and were scored as L4 larvae when the crescent-shaped immature vulval structure was observed. All *P*-values are derived from a two-sample *t*-test. *, $P<0.05$, **, $P<0.01$ compared to wild-type N2 strain.

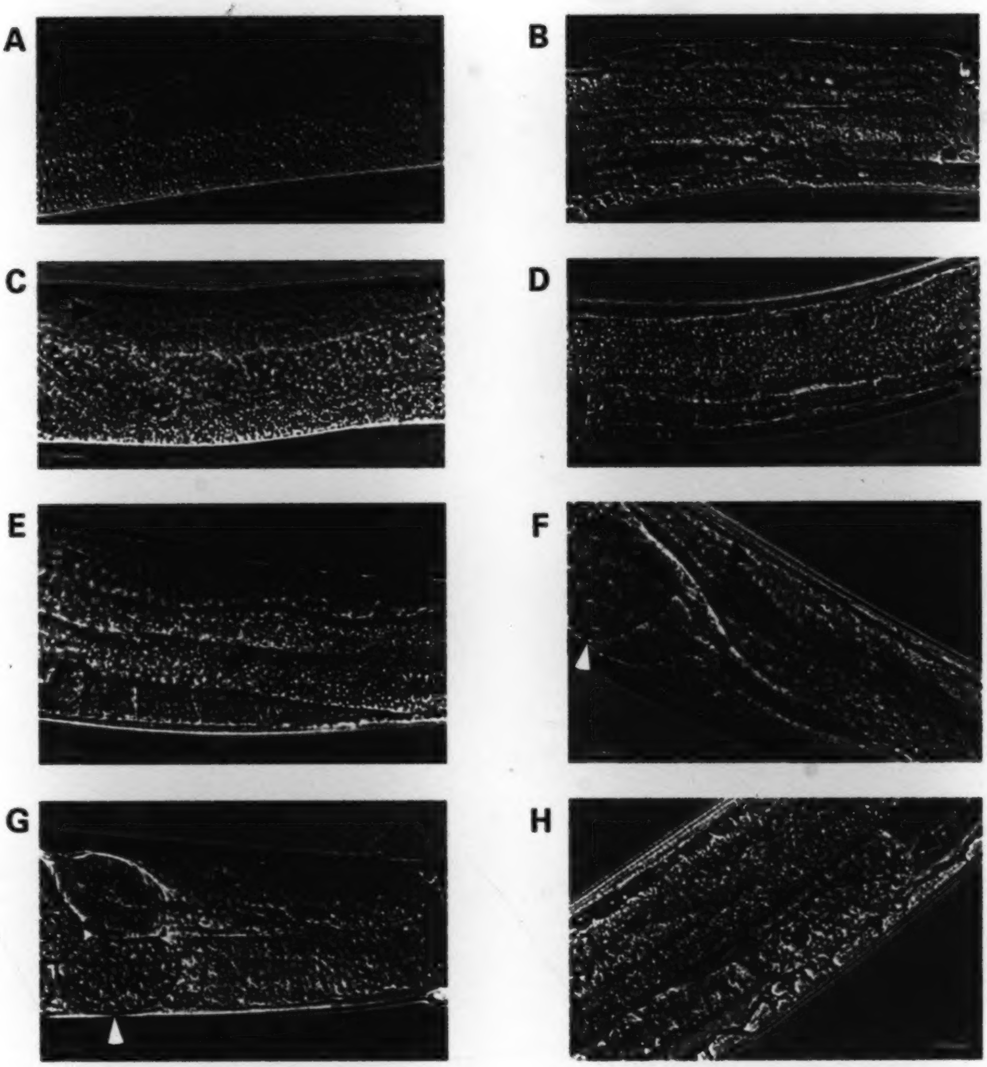


Figure 2.4 Nomarski photographs of posterior gonad arms of wild-type and transgenic mutant animals cultured at 20 °C. **(A)** N2, late L4; **(B)** A352V, late L4; **(C)** T434M, late L4; **(D)** A443F, late L4; **(E)** N2, 1 d-old adult; **(F)** A352V, 1 d-old adult; **(G)** T434M, 1 d-old adult; **(H)** A443F, 1 d-old adult. Bars = 14 μm. Syncytial germline nuclei in the gonad arms and embryos are indicated with solid and open arrows, respectively.

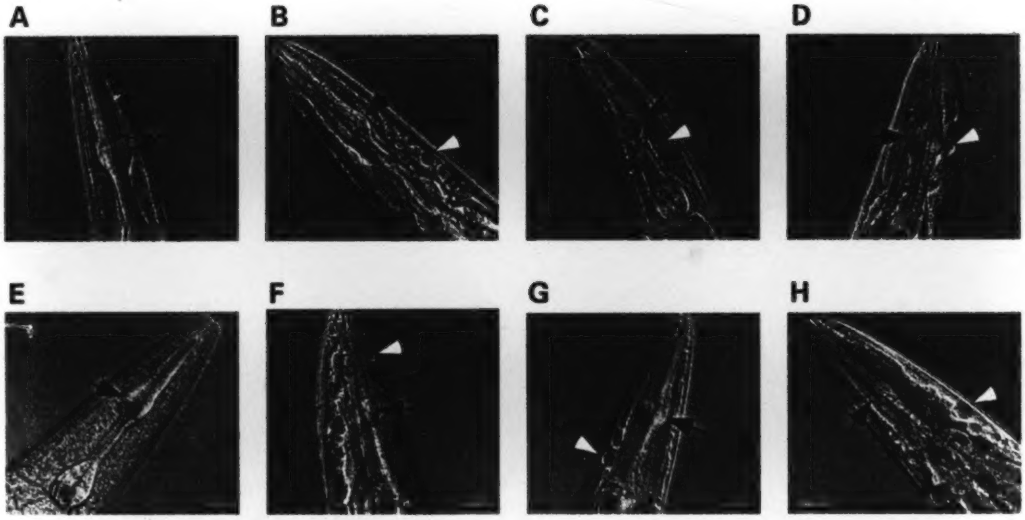


Figure 2.5 Nomarski photographs of the head regions of wild-type and mutant animals. **(A)** N2, 1 d-old adult; **(B)** A352V, 1 d-old adult; **(C)** T434M, 1 d-old adult; **(D)** A443F, 1 d-old adult; **(E)** N2, 4 d-old adult; **(F)** A352V, 4 d-old adult; **(G)** T434M, 4 d-old adult; **(H)** A443F, 4 d-old adult. Bars = 15 μm. The metacorpus of the pharynx and examples of vacuolar structures are indicated with solid and open arrows, respectively.

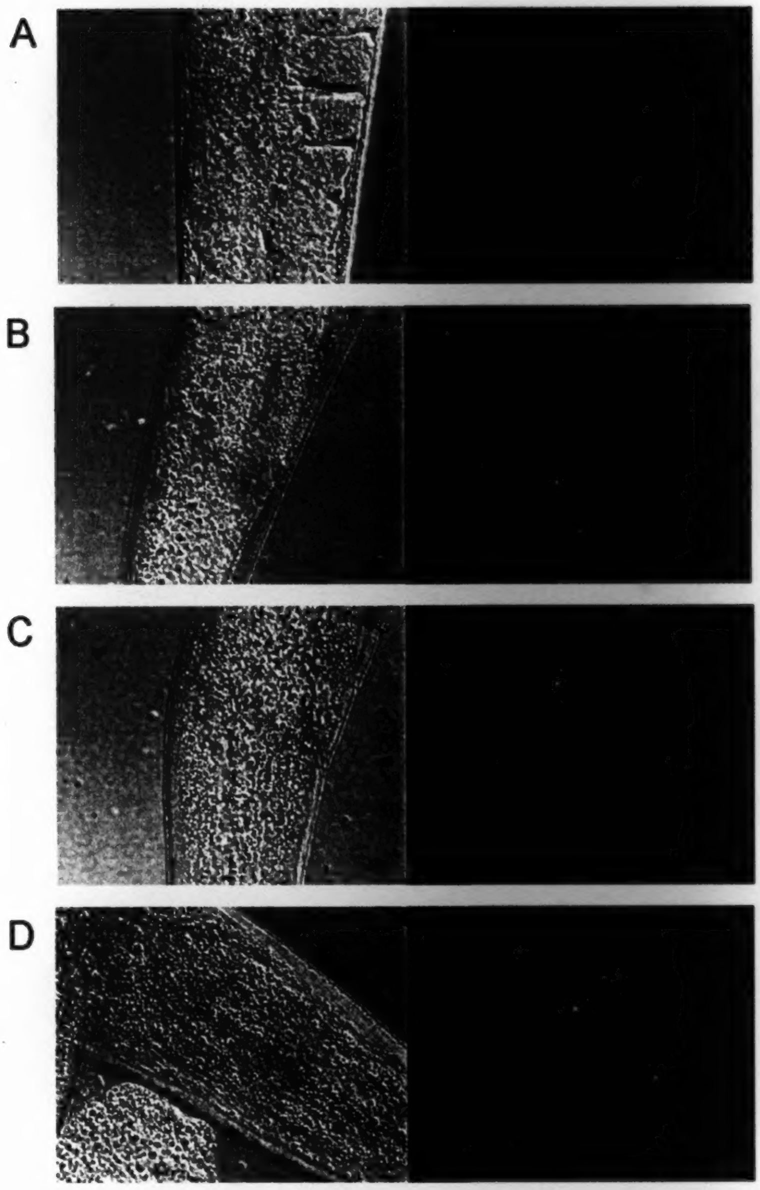


Figure 2.6 Transgenic mutants show the presence of TUNEL-positive cells. Photographs on the left are Nomarski images of the posterior gonad region of 1-d old adult hermaphrodites treated with fluorescein label using the TUNEL reaction, with the corresponding fluorescence images on the right. **(A)** N2; **(B)** N2 pre-treated with DNase I; **(C)** A352V; **(D)** A443F.

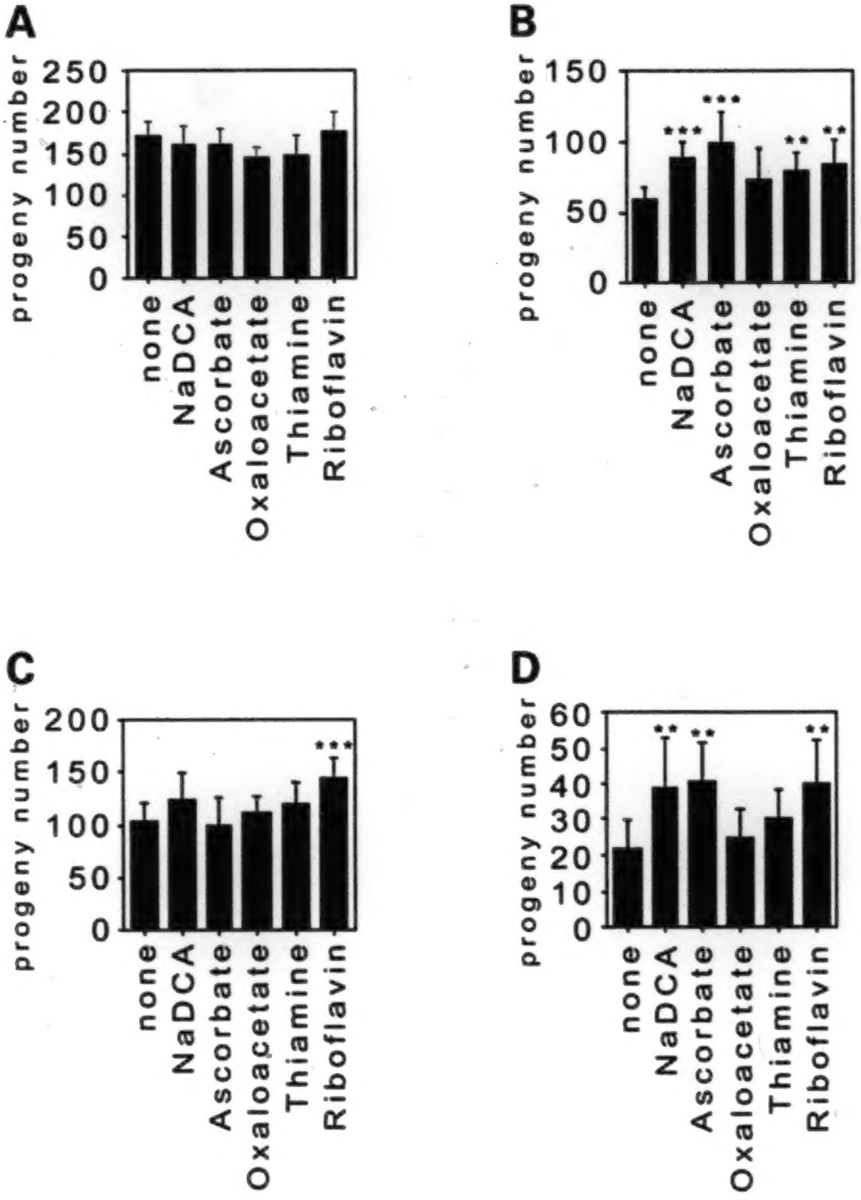


Figure 2.7 Brood size analyses of wild-type and transgenic mutant animals in the presence or absence of supplements. Only hatched progeny were scored. A minimum of 12 replicates was conducted for each strain and supplementation condition. **(A)** N2; **(B)** A352V; **(C)** T434M; **(D)** A443F. **, $P<0.01$, ***, $P<0.001$ compared to corresponding unsupplemented sample. All P-values are derived from a two-sample t-test.

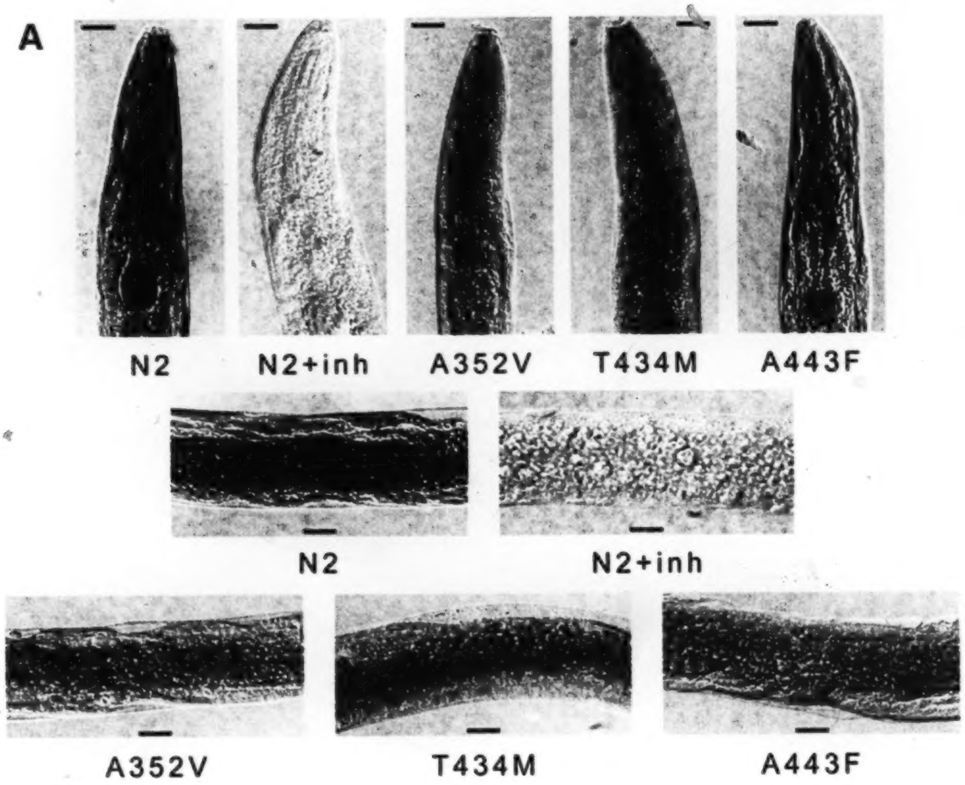
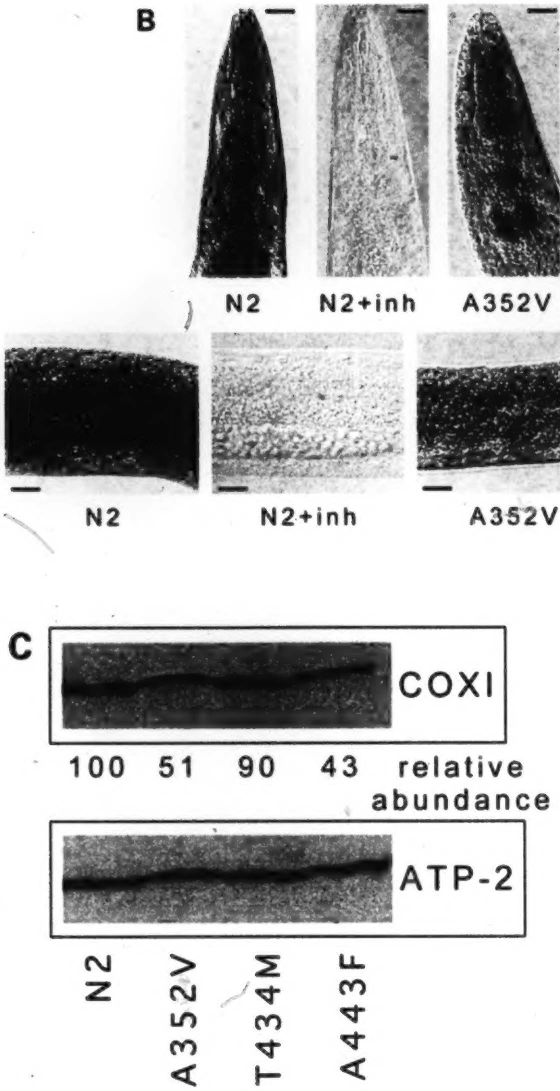


Figure 2.8 Analysis of cytochrome *c* oxidase activity and assembly in mutants and wild-type. Cytochrome *c* oxidase activity appears as a dark stain and is seen predominantly in the pharyngeal muscles and intestine, with some staining in the body wall muscles. Anterior and central regions of nematodes are shown. As a control, N2 animals were also treated with 10 mM KCN (N2 + inh) to inhibit COX activity. **(A)** Staining of L3-staged animals. Bars = 12 μm. **(B)** Staining of L4-staged animals. Bars = 15 μm. **(C)** Western blot analysis of whole-animal lysates showing relative amounts of COXI protein. Each lane represents the proteins from approximately 5,000 animals. The blot was stripped and re-probed with antiserum against ATP-2. COXI levels were quantified by densitometry and normalized to ATP-2 levels. The relative abundances of COXI are indicated.


B
N2
N2+inh
A352V
T434M
A443F
N2
N2+inh
A352V
T434M
A443F
C
COXI
100
51
90
43
relative abundance
ATP-2
N2
A352V
T434M
A443F

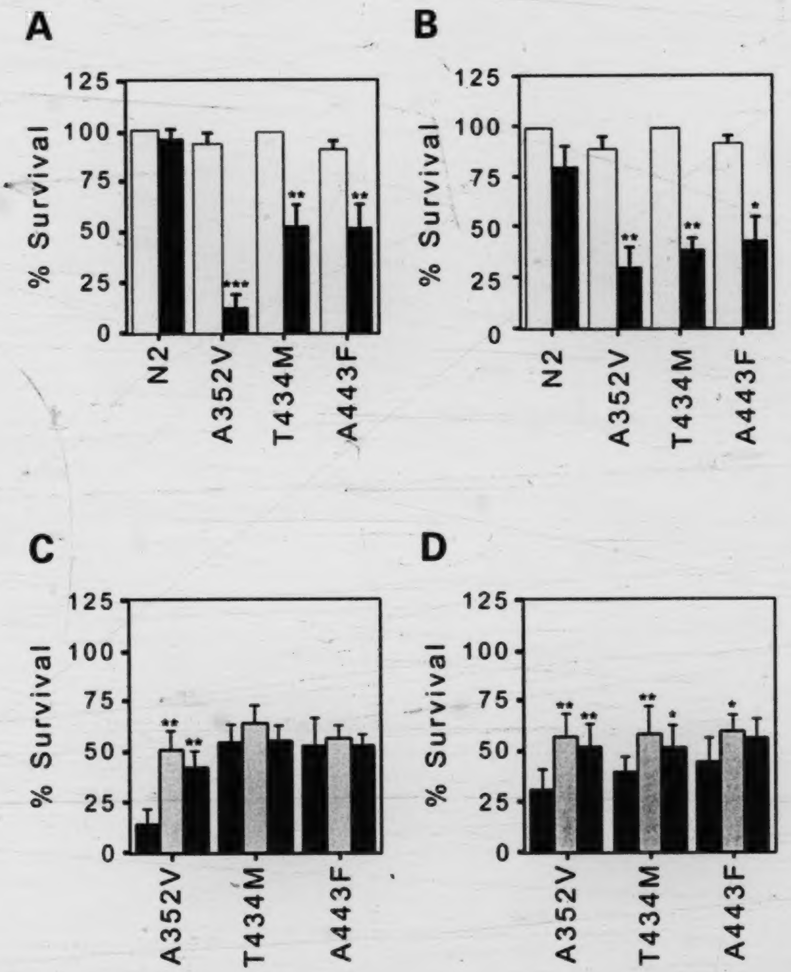


Figure 2.9 The effects of oxidative stress on wild-type and transgenic mutant strains. Survival was scored after 5 days and was defined as the percentage of L1 larvae that developed into adults. A minimum of 50 animals was scored. Each value is the mean of five trials. **(A)** L1 larvae were placed onto seeded NGM plates and incubated at 22 °C in either atmospheric (gray bars) or 100% oxygen (black bars). **(B)** L1 larvae were placed onto seeded NGM plates with no (gray bars) or 0.2 mM paraquat (black bars) and incubated at 20 °C. **(C)** L1 larvae were placed onto seeded NGM plates and incubated at 22 °C in 100% oxygen without added supplement (black bars), with 5 μg/ml sodium dichloroacetate (light gray bars), or with 1 μg/ml riboflavin (dark gray bars). **(D)** L1 larvae were placed onto seeded NGM plates with 0.2 mM paraquat without added supplement (black bars), with 5 μg/ml sodium dichloroacetate (light gray bars), or with 1 μg/ml riboflavin (dark gray bars). *P*-values were derived using two-sample *t*-test. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ compared to wild-type in **(A)** or **(B)** or to the non-supplemented control in **(C)** or **(D)**.

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Chapter 3

Riboflavin Enhances the Assembly of Mitochondrial Complex IV in *C. elegans* Complex I Mutants

3.1 Introduction

The best-known function of mitochondria is to provide cellular energy via the process of oxidative phosphorylation (OXPHOS). OXPHOS is mediated by the mitochondrial respiratory chain (MRC), which is composed of four membrane-bound electron-transporting protein complexes (I-IV) that generate a proton gradient across the mitochondrial inner membrane, and the ATP synthase (complex V) that utilizes the proton gradient for ATP generation. Defects in one or more MRC complex impairs OXPHOS and can result in mild to severe disease or even lethality. Deficiency in complex I, the NADH-ubiquinone oxidoreductase, is the most prevalent form of MRC dysfuntion (Robinson, 1998; Loeffen *et al.*, 2000; Shoubridge, 2001b; Scaglia *et al.*, 2004). Mammalian complex I is the largest respiratory chain complex, composed of at least 46 subunits, seven of which are encoded in the mitochondrial DNA (mtDNA; Attardi and Schatz, 1988; Carroll *et al.*, 2003). The complex contains a flavin mononucleotide (FMN) co-factor that serves as the entry point for electrons from NADH oxidation and up to eight iron-sulfur clusters that facilitate electron transfer through the enzyme (Walker, 1992; Ohnishi, 1998; Yano, 2002; Yagi and Matsuno-Yagi, 2003). Electron transfer from NADH to ubiquinone is coupled to vectorial proton movement across the inner membrane (Schultz and Chan, 2001). Complex I dysfunction is linked to cardiomyopathies, encephalomyopathies, and neurodegenerative disorders such as Parkinson's disease and Leigh syndrome (Robinson, 1998; Smeitink and van den Heuvel, 1999; Shoubridge, 2001b).

The exact pathophysiological mechanisms involved in most mitochondrial diseases remain poorly understood. To better describe the bioenergetic and biochemical consequences of MRC dysfunction, we developed a *Caenorhabditis elegans* model of complex I deficiency (Tsang *et al.*, 2001; Grad and Lemire, 2004). *C. elegans* complex I is composed of at least 40 subunits, many of which share very high sequence identity to their human homologues (Tsang and Lemire, 2003; Gabaldón *et al.*, 2005). Our nematode strains express missense mutations in the nuclear-encoded *nuo-1* gene, the nematode orthologue of the human *NDUFV1* gene (Grad and Lemire, 2004). The *nuo-1* and the *NDUFV1* genes encode the 51-kDa subunit of complex I, which carry the NADH-binding site, an FMN cofactor, and an iron-sulfur cluster. Patients with mutations in the *NDUFV1* gene present with a myriad of symptoms, including hypotonia, myoclonic epilepsy, brain atrophy, macrocystic leukodystrophy, acute metabolic acidosis, and Leigh syndrome (Schuelke *et al.*, 1999; Bénit *et al.*, 2001; Schuelke *et al.*, 2002). We created nematode strains expressing the single amino-acid substitutions in the NUO-1 protein: A352V, T434M, or A443F. These mutants demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent respiration. In addition, we noted specific catalytic deficiencies in complexes I and IV, particularly in the more severe A352V and A443F mutants (Grad and Lemire, 2004).

A variety of pharmacological agents have been used to treat MRC dysfunction, although there is little solid evidence supporting their use (Taylor *et al.*, 1997; Chinnery and Turnbull, 2001). Riboflavin produced significant beneficial effects, as measured by increased fertility, in all three *nuo-1* mutants (Grad and Lemire, 2004). Interestingly, riboflavin (vitamin B_2) has previously been associated with the successful treatment of complex I deficiency (Arts *et al.*, 1983; Roodhooft *et al.*, 1986; Bernsen *et al.*, 1991; Penn *et al.*, 1992). Riboflavin is a precursor to the flavin cofactors FMN and FAD (flavin adenine dinucleotide), which serve as coenzymes for numerous reactions involving one and two-electron oxidation-reduction reactions. Complex I utilizes FMN as the initial electron acceptor during the oxidation of NADH. Another important, riboflavin-dependent, metabolic reaction is catalyzed by the pyruvate dehydrogenase complex (PDHC), an FAD-containing enzyme that oxidizes pyruvate to acetyl-coenzyme A. This reaction is the major source of substrate for the Krebs cycle. If PDHC activity is low, pyruvate generated by glycolysis can accumulate and be converted to lactate, resulting in lactic acidosis. Riboflavin supplementation of complex I-deficient worms may also stimulate PDHC activity and the Krebs cycle; this is consistent with the marked attenuation of lactic acidosis by riboflavin we noted in the A443F mutant (Grad and Lemire, 2004).

Mutations leading to complex I deficiency may be localized to either nuclear or mtDNA-encoded complex I genes, or in genes encoding chaperone-like proteins that mediate the assembly of subunits and cofactors into the holoenzyme (Antonicka *et al.*, 2003; Hofhaus and Attardi, 1993; Petruzzella *et al.*, 2001; Triepels *et al.*, 2001b). In contrast, clinical deficiency of cytochrome *c* oxidase is almost never a result of mutation in one of its structural subunits, but rather is a result of an assembly factor defect (Pecina *et al.*, 2004; Shoubridge, 2001a).

To better understand the molecular bases of pathogenic complex I mutations, we have investigated the assembly and catalytic function of complexes I and IV in *C. elegans nuo-1* mutants. Here, we report assembly defects for both complexes and that these assembly defects are responsive to riboflavin, leading to enhanced catalytic performance. Thus, key mechanisms of pathogenesis for *nuo-1* mutations are the destabilization of complex I and the impaired assembly of complex IV. We suggest that the complex IV assembly defects are indirectly corrected by riboflavin supplementation, since complex IV is not a flavoprotein.

3.2 Materials and Methods

3.2.1 Strains

Worms were cultured as described (Lewis and Fleming, 1995). We used the following *C. elegans* strains: N2 (Bristol) wild type; LB25, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx25*[p016bA352V]; LB26, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaIs26*[p016bT434M]; LB27, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx27*[p016bA443F] (Grad and Lemire, 2004). Worms cultured in liquid medium were supplemented with 1 μg/ml riboflavin with additional riboflavin added every second day until harvested.

3.2.2 Electrophoresis and Western Blot Analyses

Mitochondria were isolated as previously described (Grad and Lemire, 2004). Fifty μg of mitochondrial protein were solubilized in gel-loading buffer and resolved by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% or 12% acrylamide) (Tsang *et al.*, 2001). Proteins were transferred electrophoretically to nitrocellulose or polyvinylidene fluoride membranes. Blots were treated with rabbit polyclonal antisera against the bovine 51-kDa subunit (a gift from Dr. M. Yamaguchi, USA), the *N. crassa* TYKY subunit (a gift from Dr. F. Nargang, Canada), or the *S. cerevisiae* Atp-2p (Dibrov *et al.*, 1998). Mouse monoclonal antisera against the human NDUFS3 protein (30-kDa complex I subunit) or COXI (MitoSciences, Eugene, Oregon) were also used. For development, blots were treated with peroxidase-labeled goat anti-rabbit or goat anti-mouse secondary antibodies. The Enhanced Chemiluminescence Western Blotting System (Amersham Biosciences, Buckingham, UK) or the Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Inc., Rockford, IL) were used for detection. Signal quantification was performed with the BioRad Gel Doc

1000 Image Analysis System and Molecular Analysts software (BioRad Laboratories, Hercules, CA).

3.2.3 Native Gel Electrophoresis and Histochemical Staining

Blue native gradient gels (4-13% for complex I analysis; 5-15% for complex IV analysis) were loaded with 300 μg mitochondrial protein as described (Schägger and von Jagow, 1991). Following electrophoresis, NADH dehydrogenase activity was detected by incubating gels in 20 ml 50 mM Tris-HCl, pH 7.4 containing 0.5 mM tetranitroblue tetrazolium and 5 mM NADH at 37 °C for 80 min in the dark with gentle rocking. For the detection of cytochrome *c* oxidase activity, gels were incubated in 20 ml 50 mM Tris-HCl, pH 7.4 containing 0.1% 3,3'-diaminobenzidine, 0.1% cytochrome *c*, 0.02% catalase at 37 °C for 90 min in the dark with gentle rocking. Gels used for Western blot analysis were incubated in 20 mM Tris-base, 150 mM glycine, 20% (v/v) methanol, 0.08% (w/v) SDS for 10 min before electrophoretic transfer to Immobilon-P membranes (Millipore Corp., Billerica, MA; Dekker *et al.*, 1997). Following transfer, excess stain was removed as described (Nijtmans *et al.*, 2002).

3.2.4 MRC Assays

Enzymatic activities were measured on an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK) as described (Birch-Machin and Turnbull, 2001). Rotenone-sensitive, NADH-decylubiquinone oxidoreductase activity was measured at 340 nm using 65 μM 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone as electron acceptor, 2 μg/ml antimycin A, 0.13 mM NADH. Rotenone was added to 2 μg/ml. Cytochrome *c* oxidase activity was measured at 550 nm using 15 μM cytochrome *c* as electron acceptor in the presence of 0.45 mM lauryl maltoside. Potassium cyanide was added to 1 mM.

3.3 Results

3.3.1 Steady-state Levels of Complexes I and IV are Reduced in nuo-1 Mutants

We investigated the steady-state levels of complexes I and IV in mitochondria isolated from wild-type *C. elegans* and from three complex I-deficient strains: LB25, LB26, and LB27. LB25 and LB27 carry extrachromosomal transgenic arrays expressing the A352V and A443F *nuo-1* alleles, respectively. LB26 carries an integrated transgene encoding the T434M *nuo-1* mutation. The relative abundance of three complex I subunits was determined by Western blot analysis. We observed severe reductions in the amounts of NUO-1 protein (51-kDa subunit), moderate reductions in the amounts of 30-kDa subunit, but no reduction in the amounts of TYKY (23-kDa) subunit in the mutants (Figure 3.1, Table 3.1). The steady-state level of the mtDNA-encoded COXI subunit of complex IV also showed a moderate reduction. No differences were observed in the steady-state levels of the complex V subunit, ATP-2. The *nuo-1* mutations differentially affect the steady-state levels of individual complex I subunits and affect the levels of at least one subunit in complex IV.

We investigated the effects of riboflavin supplementation on the steady-state levels of the complex I and complex IV subunits in the strains carrying the two most severe alleles, LB25 and LB27. Mitochondria from the riboflavin-supplemented strains showed significant increases in the relative abundance of NUO-1 protein, increasing to 74% and 72% of wild type, respectively (Figure 3.1, Table 3.2). Surprisingly, COXI levels were also elevated by supplementation with riboflavin, increasing to 85% and 83% of wild type, respectively (Figure 3.1, Table 3.2). The steady-state levels of the 30-kDa and 23-kDa complex I subunits and of the ATP-2 subunit were not affected by riboflavin. These data indicate that riboflavin differentially affects the steady state-levels of individual complex I subunits. In addition, riboflavin modulates the levels of COXI, a subunit of complex IV, which does not contain a flavin cofactor.

3.3.2 nuo-1 Mutations Impair MRC Complex Assembly

To determine how the reduced steady-state levels of MRC subunits affect the amounts of fully assembled complexes, we resolved mitochondrial proteins by blue native gel electrophoresis (BN-PAGE). The gels were stained for NADH dehydrogenase activity, an activity that is present in complex I holoenzyme and in some sub-complexes (Ugalde *et al.*, 2004a). A species with high levels of NADH dehydrogenase activity that migrates with an apparent molecular weight of ~950-kDa was detected in wild-type mitochondria; it most likely corresponds to complex I holoenzyme (Figure 3.2A). Much lower levels of NADH dehydrogenase activity are associated with the 950-kDa species in mitochondria from LB25, LB26, or LB27. In addition, LB25 and LB26 mitochondria contain complexes with significant NADH dehydrogenase activity migrating at ~640-kDa and ~600-kDa, respectively. These smaller species are absent from wild-type mitochondria. LB27 mitochondria do not contain detectable NADH-dehydrogenase activity in the 600-640-kDa range. In mitochondria from riboflavin-supplemented LB25 and LB27, there is a significant increase in the amount of NADH dehydrogenase activity at ~640 kDa (Figure 3.2A). However, riboflavin supplementation does not increase the activity associated with the ~950-kDa species.

To confirm that the high molecular weight NADH dehydrogenase-active species are in fact related to complex I, we used Western blot analysis with an antiserum directed against the bovine 51-kDa subunit, which cross-reacts with *C. elegans* NUO-1. In wild-type mitochondria, we detected a strong but diffuse signal centered at ~1-MDa, which corresponds closely to the position of the ~950-kDa NADH dehydrogenase-active species (Figure 3.2B). This confirms that the *C. elegans* complex I is ~950 kDa in size, similar to mammalian complex I. Immunologically-detectable, high molecular weight species containing NUO-1 are considerably less abundant in LB26 and almost absent in LB25 and LB27. This is in agreement with the steady-state levels of NUO-1 determined by Western blot analysis after denaturing gel electrophoresis (Figure 3.1). The diffuse NUO-1-specific signal in LB26 mitochondria is shifted towards smaller molecular weights, being centered at ~800-kDa. This suggests the ~800-kDa signal either represents a complex I sub-complex or that the holoenzyme is unstable during electrophoresis. Mitochondria from riboflavin-supplemented LB25 and LB27 demonstrate significant increases in NUO-1-specific signal. The NUO-1-specific signal in these samples is even more diffuse and is found as far down as ~550-kDa (Figure 3.2B). The signal by Western blot overlaps with the position of the ~640-kDa species seen by NADH dehydrogenase activity staining, strongly suggesting that this species is a NUO-1-containing assembly intermediate of complex I.

We also investigated the assembly of complex IV in the *nuo-1* mutants. Mitochondrial proteins were separated by blue native gel electrophoresis and the gels stained for cytochrome *c* oxidase activity. Wild-type mitochondria contain five distinct cytochrome *c* oxidase-active species (Figure 3.3). A major species with an apparent molecular weight of 210-kDa, likely representing monomeric complex IV, appears first during the staining reaction. Minor species of 130-, 165-, 270-, and 420-kDa, the latter likely representing dimeric complex IV, appear later during the in-gel staining reaction.

Mitochondria from the LB25, LB26, and LB27 mutants all produce different staining patterns. The 130-, 210-, and 270-kDa species are present in all three mutants, although their staining intensities are variably reduced compared to wild type. Complex IV dimer (420-kDa species) is only weakly detected in LB25 and LB27 mitochondria and appears absent in LB26. The 165-kDa species is absent in all three mutants; instead, an alternate species with an apparent molecular weight of ~160-kDa is present. Finally, a smaller additional species at ~120-kDa is present in the mutants but not in wild-type.

We further examined the effects of riboflavin on the assembly of complex IV because the mutant strains showed riboflavin-responsive increases in the steady-state level of COXI (Figure 3.1). The most obvious riboflavin-dependent effect is the disappearance of the 120-kDa species from the mutants, making the cytochrome *c* oxidase staining patterns more closely resemble that of wild-type. However, the 160-kDa species remains intact, as opposed to the 165-kDa species seen in the wild-type. The overall cytochrome *c* oxidase staining intensities of the mutant mitochondria are consistently increased by riboflavin, suggesting complex IV assembly and/or stability is enhanced by the vitamin. Our analysis has revealed the presence of aberrant cytochrome *c* oxidase complexes in *nuo-1* mutants and the abundance of some of these is responsive to riboflavin supplementation.

3.3.3 Riboflavin Increases the Activities of Complexes I and IV

The in-gel activity staining suggests that riboflavin increases the amounts of assembled, active complexes I and IV. However, the in-gel assays are only semi-quantitative. We measured the rotenone-sensitive, NADH-decylubiquinone reductase activities in LB25 and LB27 mitochondria; as reported previously, these are approximately 30% of the wild-type (Figure 3.4A; Grad and Lemire, 2004). Mitochondria isolated from riboflavin-supplemented cultures contain more than twice the amount of complex I activity (Figure 3.4A). Likewise, the cyanide-sensitive cytochrome *c* oxidase activities of LB25 and LB27 mitochondria are ~50% of wild type levels (Figure 3.4B). The activities also increase substantially when the strains are cultured in the presence of riboflavin.

3.4 Discussion

We have modeled pathogenic human complex I mutations in *C. elegans* in an effort to elucidate the biochemical and bioenergetic mechanisms by which MRC dysfunction leads to disease. Our previous study suggested that mutations in complex I cause pathology in at least three ways: 1) A reduced ability to oxidize NADH perturbs the redox balance of the cell and impairs other metabolic pathways. 2) There is an acceleration of the rate of oxygen free-radical generation that leads to oxidative stress. 3) Impaired OXPHOS results in an energy deficit. In this work, we have extended our understanding of these complex I mutations by investigating their effects on the assembly and activity of complexes I and IV. In addition, we have revealed an unexpected effect of riboflavin on the assembly of the cytochrome *c* oxidase complex.

A key result of our investigation is the clear demonstration of reduced levels of complex I holoenzyme in *nuo-1* mutants. Similar observations have been reported for human complex I mutations affecting a variety of nuclear or mtDNA-encoded subunits (Hofhaus and Attardi, 1993; Triepels *et al.*, 2001a; Antonicka *et al.*, 2003; Scacco *et al.*, 2003; Ugalde *et al.*, 2003; Ugalde *et al.*, 2004a). Of the complex I subunits we investigated, the NUO-1 protein itself showed the greatest reduction in steady-state levels. The A352V, T434M, and A443F mutations we introduced into the *nuo-1* gene affect conserved residues. We believe these residues are important for protein folding and that newly-synthesized mutant NUO-1 protein is more often degraded than assembled into the holoenzyme. The A352, T434, and A443 residues are not located in the cofactor-binding motifs identified by sequence analysis, but we do not have sufficient high resolution structural information to eliminate a role for them in cofactor binding (Walker, 1992).

Unlike bovine or human complex I, there is no information on the localization of specific subunits in the *C. elegans* complex I holoenzyme. There is, however, mounting evidence that mammalian complex I is assembled from a series of modules or sub-complexes (Friedrich and Böttcher, 2004). Both the 30-kDa and the 23-kDa subunits are located within the peripheral arm of complex I and more specifically with the Iλ fraction, the same sub-complex that contains the 51-kDa subunit (Carroll *et al.*, 2003; Ugalde *et al.*, 2004b). The mutations in *C. elegans nuo-1* affect the steady-state level of the 30-kDa subunit, but not of the 23-kDa subunit. This suggests that the stability or assembly of certain sub-complexes is compromised in the mutants, leading to the loss of specific subunits. Very little is known about the 30-kDa subunit, as it contains no redox cofactors, while the 23-kDa subunit is believed to house two iron-sulfur clusters (Walker, 1992). Models for the assembly of mammalian complex I have been proposed, but the models are incomplete or insufficiently detailed to allow us to suggest why the levels of 30-kDa and 23-kDa subunits are differentially affected by the *nuo-1* mutations (Antonicka *et al.*, 2003; Ugalde *et al.*, 2004b; Vogel *et al.*, 2004). Due to the small number of available antibodies against *C. elegans* complex I subunits, more detailed investigations into the assembly and localization of NUO-1 are severely restricted.

We recognized that the NADH dehydrogenase activity stain and corresponding α-NUO-1-specific signal (Figure 3.2A, B) do not always coincide in intensity or location for some *nuo-1* mutants. It should be noted that each assay visualizes complex I assembly at a different temporal stage and that variability in complex I stability in *nuo-1* mutants over time may account for random differences in activity staining and/or α-NUO-1-specific signal intensity and location. This could also explain similar inconsistencies between the intensity of NADH dehydrogenase activity staining and the levels of rotenone-sensitive NADH-decylubiquinone oxidoreductase activity (Figure 3.4A) in different *nuo-1* mutant mitochondria. It should also be noted that the two assays measure NADH oxidation in fundamentally different manners, thus their results might not always coincide.

With respect to complex I subunits, the beneficial effects of riboflavin are limited to the NUO-1 protein. This is perhaps not surprising since it is the NUO-1 subunit that carries the FMN cofactor and it is well known that ligands can promote protein folding or stabilize folded proteins. Supplementation with riboflavin likely results in increased availability of FMN leading to enhanced rates of NUO-1 folding to a more stable cofactor-bound form and of assembly into stable sub-complexes. The appearance of lower molecular weight assembly intermediates and the continued low abundance of 30-kDa subunit in riboflavin-supplemented mutant mitochondria indicate that stabilizing and assembling NUO-1 into sub-complexes is not sufficient to entirely correct the assembly defects. It does emphasize the importance of further studying the assembly pathways of MRC enzymes for diagnosing and treating mitochondrial disease.

Although we have identified aberrant assembly of complex IV as contributing to the *nuo-1* mutant phenotypes, we have not determined the mechanism by which this occurs. Patients with mutations in the NDUFS2 and NDUFS4 subunits of complex I presented with a decreased level of complex III dimer (Ugalde *et al.*, 2004a). These observations suggest that a physical interaction between complexes I and III may exist. This is supported by genetic evidence where a mutation in cytochrome *b* of complex III produces a combined deficiency in complexes I and III (Lamantea *et al.*, 2002). A supercomplex comprising complexes I and III and up to four copies of complex IV has been detected in mammalian mitochondria (Schägger and Pfeiffer, 2000). In fact, almost all the complex I was found associated with complex III. Our previous investigations did not identify a consistent effect of the *nuo-1* mutations on complex III activity (Grad and Lemire, 2004). Under certain detergent conditions, a direct interaction between complexes I and IV without complex III was detected (Schägger and Pfeiffer, 2000). A combined deficiency in complexes I and IV has been reported but the precise molecular lesion is unknown (Roodhooft *et al.*, 1986). Complex I deficiency may destabilize complex IV by preventing or diminishing the formation of supercomplexes. Alternatively, complex I dysfunction may indirectly affect assembly of complex IV through altered expression of complex IV subunits, including isoforms, or of complex IV assembly factors. These altered expression patterns may account for the relatively small size differences seen in complex IV species by BN-PAGE. Finally, complex I deficiency may result in a lower membrane potential across the mitochondrial inner membrane, which is necessary for complex IV assembly (Herrmann *et al.*, 1995). Riboflavin likely exerts its beneficial effects on complex IV indirectly, by enhancing complex I assembly or stability.

In conclusion, we have shown that the catalytic deficiencies of complexes I and IV observed in *C. elegans nuo-1* mutants are due to their impaired assembly or stability. NUO-1 mutants have reduced levels of fully assembled complex I, and lower molecular weight assembly intermediates. Riboflavin supplementation stabilizes the steady-state levels of NUO-1, thereby increasing the abundance of fully assembled complex I and of specific assembly intermediates. NUO-1 mutants also demonstrate aberrant complex IV assembly patterns, which are partially corrected by riboflavin. We suggest that the effects of riboflavin are profound because this vitamin improves the activities of both complex I and IV, which in turn leads to reduced oxidative stress and lactic acidosis and increased metabolic function.

Table 3.1 Relative steady-state levels of MRC proteins.

Protein	N2	LB25	LB26	LB27
NUO-1	1.00	0.29 ± 0.05*	0.75 ± 0.07*	0.45 ± 0.04*
23-kDa	1.00	0.97 ±0.03	0.99 ± 0.02	0.97 ± 0.02
30-kDa	1.00	0.71 ± 0.02*	0.84 ± 0.02*	0.72 ± 0.05*
COXI	1.00	0.58 ± 0.03*	0.86 ± 0.04*	0.66 ± 0.03*
ATP-2	1.00	1.00 ± 0.02	1.01 ± 0.02	1.00 ± 0.03

Reported values are normalized to levels in the wild-type N2. Values are means ± SD of a minimum of three experiments. *, $P<0.05$ using two-sample *t*-test, compared to N2 wild-type value.

Table 3.2 Effects of riboflavin on protein steady-state levels.

Protein	LB25	LB25+Rb	LB27	LB27+Rb
NUO-1	0.29 ± 0.05	0.74 ± 0.04*	0.45 ± 0.04	0.72 ± 0.03*
23-kDa	0.97 ±0.03	0.98 ± 0.02	0.97 ± 0.02	0.99 ± 0.01
30-kDa	0.71 ± 0.02	0.73 ± 0.01	0.72 ± 0.05	0.76 ± 0.04
COXI	0.58 ± 0.03	0.85 ± 0.04*	0.66 ± 0.03	0.83 ± 0.04*
ATP-2	1.00 ± 0.02	1.02 ± 0.05	1.00 ± 0.03	0.99 ± 0.01

Reported values are normalized to levels in the wild-type N2. Values are means ± SD of a minimum of three experiments. *, $P<0.05$ using two-sample *t*-test, compared to the corresponding unsupplemented strain.

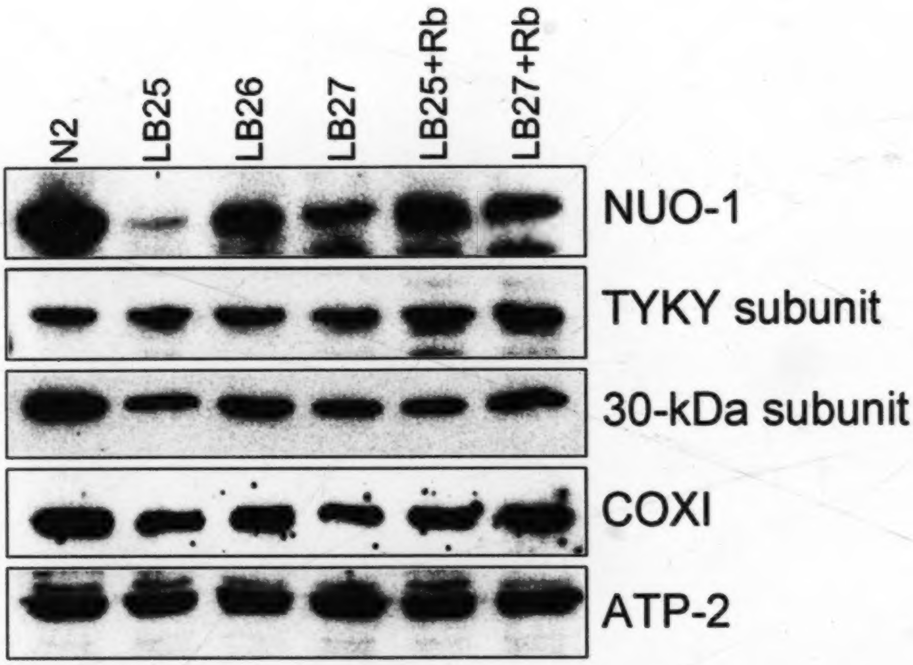

N2
LB25
LB26
LB27
LB25+Rb
LB27+Rb
NUO-1
TYKY subunit
30-kDa subunit
COXI
ATP-2

Figure 3.1 Steady-state levels of MRC proteins are diminished in mutant mitochondria. 50 μg of isolated mitochondria from the wild-type (N2) or the *nuo-1* mutants LB25-LB27 were loaded into each lane and specific subunits of complexes I, IV, and V were detected by Western blot analysis. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin.

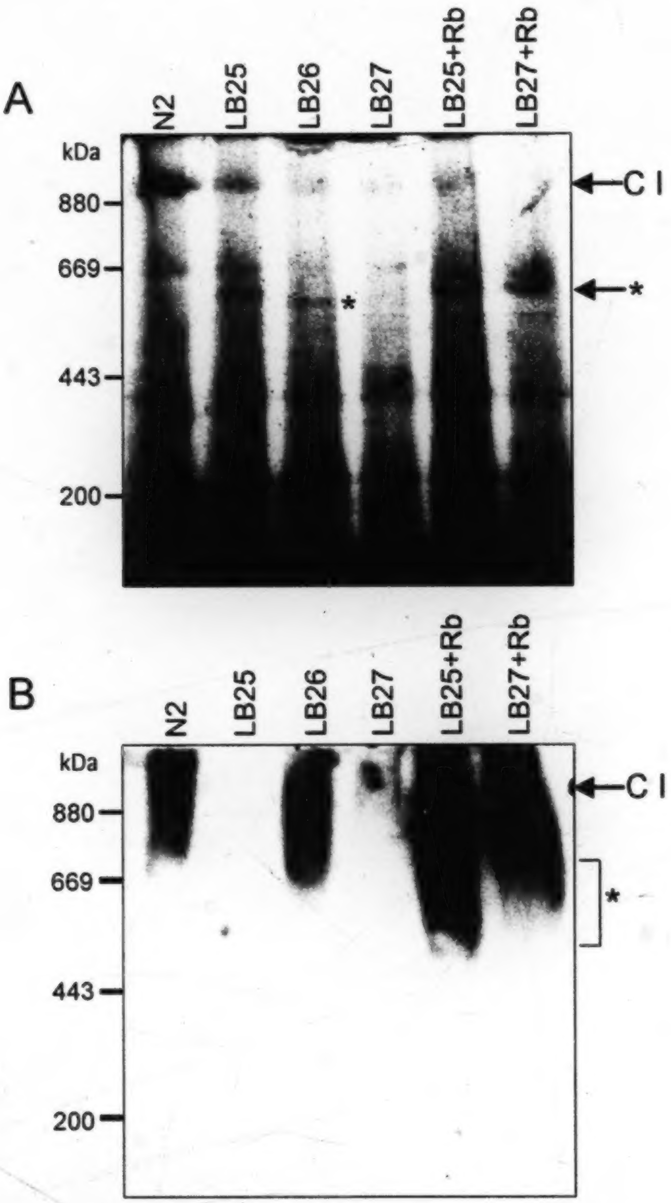
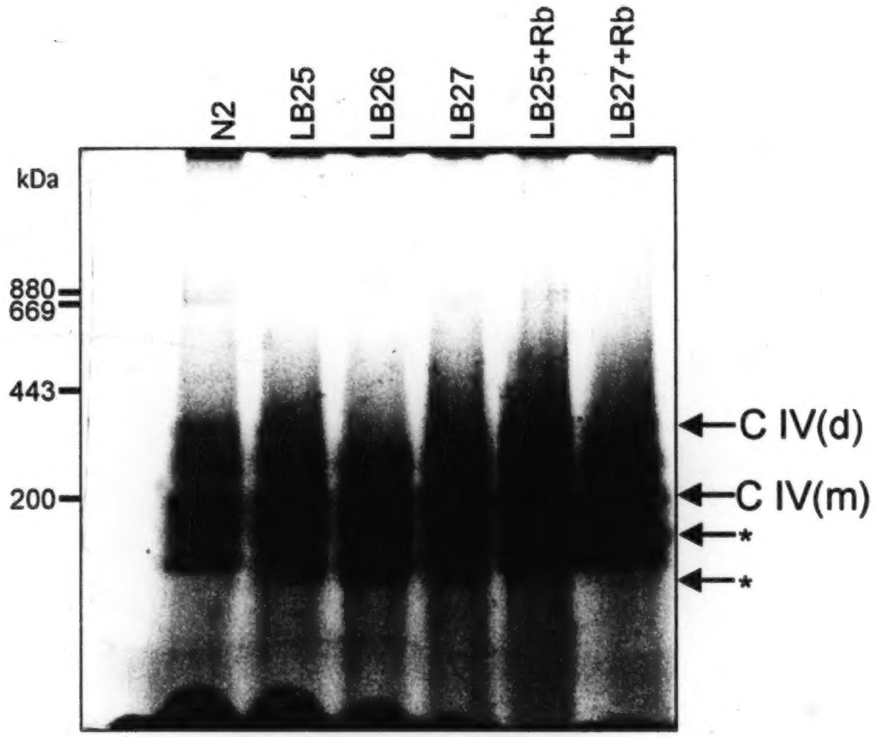

A
N2
LB25
LB26
LB27
LB25+Rb
LB27+Rb
kDa
880
669
443
200
C I
*
*
B
N2
LB25
LB26
LB27
LB25+Rb
LB27+Rb
kDa
880
669
443
200
C I
*

Figure 3.2 Complex I assembly is impaired in *nuo-1* mutant mitochondria. (A) Mitochondrial proteins (300 μg) were resolved by BN-PAGE and the gel stained for NADH dehydrogenase activity. **(B)** Mitochondrial proteins were resolved by BN-PAGE and the gel was transferred for Western blot analysis with polyclonal antiserum against the bovine 51-kDa subunit of complex I. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin; C I, fully assembled complex I holoenzyme; *, mutant-specific assembly intermediates.



N2
LB25
LB26
LB27
LB25+Rb
LB27+Rb
kDa
880
669
443
200
C IV(d)
C IV(m)
*
*

Figure 3.3 Complex IV assembly is impaired in *nuo-1* mutant mitochondria. Mitochondrial proteins (300 μg) were resolved by BN-PAGE and the gel stained for cytochrome *c* oxidase activity. Rb, mitochondria were isolated from strains cultured in the presence of 1 μg/ml riboflavin; C IV(m), monomeric complex IV; C IV(d), dimeric complex IV; *, mutant-specific assembly intermediates.

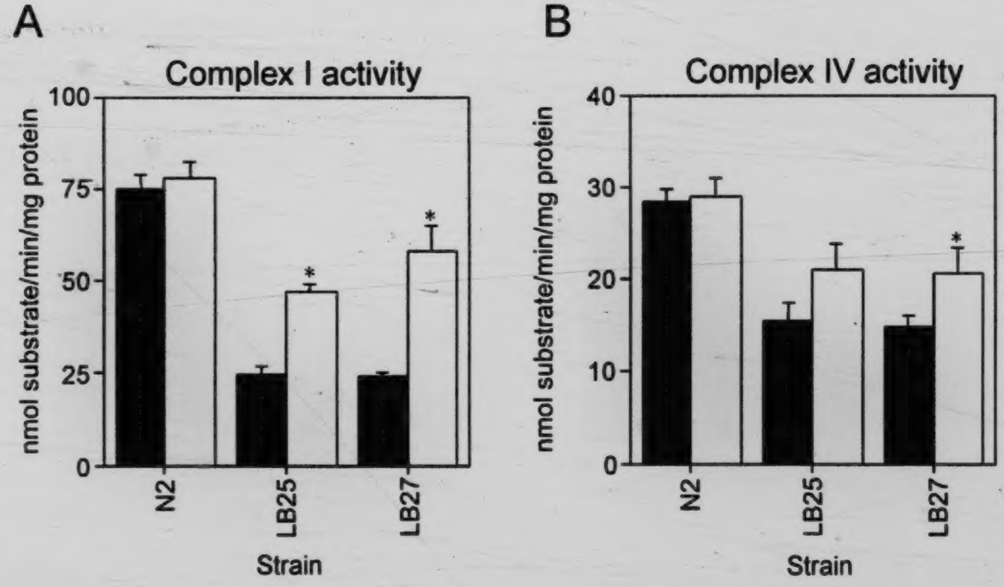


Figure 3.4 Riboflavin increases the enzyme activities of complexes I and IV in *nuo-1* mutants. **(A)** Complex I activity was measured as the rotenone-sensitive NADH-decylubiquinone oxidoreductase activity. **(B)** Complex IV was measured as the cyanide-sensitive oxidation of reduced cytochrome *c*. Reported values are means of four independent trials. Black bars represent activities in mitochondria of strains grown in the absence of riboflavin; white bars represent activities of strains cultured in the presence of 1 μg/ml riboflavin. *, $P<0.05$ compared to corresponding unsupplemented sample using a two-sample *t*-test.

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Chapter 4

A New Pathway for Lactate Oxidation in the Treatment of Lactic Acidosis and Mitochondrial Dysfunction in Complex I-deficient Mutants of *C. elegans*

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4.1 Introduction

Mitochondrial dysfunction occurs with an estimated incidence of 1 in 10,000 live births and is often caused by a deficiency in complex I, the NADH-ubiquinone oxidoreductase (Meulemans *et al.*, 2004; Robinson, 1998; Scaglia *et al.*, 2004; Triepels *et al.*, 2001). Mammalian complex I, embedded in the mitochondrial inner membrane, is the largest respiratory chain complex, composed of at least 46 subunits, seven of which are encoded in the mitochondrial DNA (mtDNA; Carroll *et al.*, 2003; Gabaldón *et al.*, 2005). The complex contains a flavin mononucleotide (FMN) cofactor, which serves as the first electron acceptor upon NADH oxidation. It also contains up to eight iron-sulfur clusters that mediate electron transfer through the enzyme to ubiquinone, the final electron acceptor (Walker, 1992; Yagi and Matsuno-Yagi, 2003; Yano, 2002). Electron transfer from NADH to ubiquinone is coupled to proton pumping across the mitochondrial inner membrane and the formation of a proton gradient that is utilized by the ATP synthase (complex V) to generate ATP. Complex I dysfunction is associated with myopathies, encephalomyopathies, and neurodegenerative disorders such as Parkinson's disease and Leigh syndrome (Robinson, 1998; Shoubridge, 2001; Smeitink *et al.*, 1998; Triepels *et al.*, 2001). Currently, there are no cures for diseases resulting from mitochondrial respiratory chain dysfunction (DiMauro *et al.*, 2004; Smith *et al.*, 2004).

To better understand the molecular mechanisms of pathogenesis of mitochondrial dysfunction, we developed a *Caenorhabditis elegans* model of complex I deficiency (Grad and Lemire, 2004). The *nuo-1* gene is the nematode ortholog of the human *NDUFV1* gene; they encode the 51-kDa subunit of complex I, which forms the active site for NADH oxidation and contains FMN and iron-sulfur cluster cofactors (Tsang *et al.*, 2001). Modified *nuo-1* genes containing the missense mutations A352V, T434M, or A443F were introduced into a nematode strain with a *nuo-1* deletion background (Grad and Lemire, 2004). The resulting transgenic strains demonstrate hallmark features of complex I dysfunction such as lactic acidosis and decreased NADH-dependent mitochondrial respiration. The lactic acidosis can be significantly alleviated with the pharmacological agents, riboflavin or sodium dichloroacetate. Sodium dichloroacetate is an activator of the pyruvate dehydrogenase complex and stimulates the entry of pyruvate into the Krebs cycle. Riboflavin, or vitamin B_2 is a precursor for the flavin cofactors FMN and FAD and may stimulate complex I and the pyruvate dehydrogenase complex, which are both flavoproteins. Riboflavin and sodium dichloroacetate produce significant improvements in mutant fitness, suggesting that the redox imbalance and subsequent lactic acidosis are key pathogenic mechanisms responsible of complex I-deficiency.

Complex I is the primary route for the oxidation of NADH generated by glycolysis and the Krebs cycle in aerobic cells. NADH accumulates when complex I activity is impaired (Munnich and Rustin, 2001). Excess NADH inhibits glycolysis and the Krebs cycle as well as the pyruvate dehydrogenase complex, resulting in defective energy metabolism and the build-up of pyruvate in the cell. Cytosolic lactate dehydrogenases use NADH to reduce pyruvate to lactate resulting in hyperlactataemia and lactic acidosis, which can directly cause malaise, muscle weakness, exercise intolerance, and vomiting (Bénit *et al.*, 2001; Chinnery and Turnbull, 2001; Schuelke *et al.*, 1999; Wallace, 1994). Therefore, the reaction catalyzed by lactate dehydrogenase directly relates the ratio of lactate to pyruvate to the ratio of NADH to NAD^+ (Robinson *et al.*, 1990). Chronic redox imbalance likely modulates gene expression and may account for the often progressive nature of mitochondrial diseases (Guarente, 2000; Lane, 2003; Starai *et al.*, 2002; Wallace, 1994). Addressing the redox imbalance and the lactic acidosis in mitochondrial disease states is likely to elicit both short- and long-term benefits.

Strategies to directly correct complex I deficiencies using gene therapy are impractical for at least three reasons. First, mammalian complex I requires the expression of 46 subunit-encoding genes, and a variety of mutations have been identified in many of these subunits (Carroll *et al.*, 2003; DiMauro and Hirano, 2005). Gene therapy with wild-type versions of subunit-encoding genes would require the development of numerous expression constructs, each specific for a small number of mutations. Second, seven subunits of complex I are encoded in the mtDNA, which is currently not easily accessible for transformation or for direct manipulation. Allotopic expression of mtDNA-encoded subunits presents its own technical challenges (Schon and DiMauro, 2003). Finally, many patients with complex I deficiencies do not have mutations in subunit-encoding genes, implying that assembly factors specific for complex I biogenesis may be affected in these cases (Shoubridge, 2001).

Gene therapy strategies for complex I deficiency have focused on replacing the functionality of complex I rather than on correcting the enzyme itself. The *Saccharomyces cerevisiae NDI1* gene encodes a single-subunit NADH dehydrogenase bound to the matrix side of the mitochondrial inner membrane that oxidizes NADH and reduces ubiquinone (de Vries and Marres, 1987). Ndi1p does not, however, couple NADH oxidation to proton pumping and thus, can not fully replace complex I (de Vries and Grivell, 1988). When expressed in respiration-deficient Chinese hamster or human cell lines, Ndi1p could fully restore NADH-dependent respiration and the ability to grow on galactose (Bai *et al.*, 2001; Seo *et al.*, 1998; Seo *et al.*, 1999). *NDI1* has been efficiently and functionally expressed in non-dividing human cells (Seo *et al.*, 2000) and in dopaminergic neuronal cells using a recombinant adeno-associated virus vector (Seo *et al.*, 2002). Recently, *NDI1* was successfully expressed *in vivo* in the skeletal muscle and brains of rodents, with sustained expression observed for at least seven months (Seo *et al.*, 2004). These data suggest that gene therapy approaches may be useful tools for the treatment of encephalomyopathies and neurodegenerative diseases caused by complex I deficiency.

In this study, we have developed an alternative gene therapy strategy using the *S. cerevisiae CYB2* gene. *CYB2* encodes a homotetrameric heme and FMN-containing L-lactate-cytochrome *c* oxidoreductase, known as cytochrome *b*2 (Mowat and Chapman, 2000). Cyb2p is normally located in the yeast mitochondrial intermembrane space where it oxidizes lactate to pyruvate and directly reduces cytochrome *c* (de Vries and Marres, 1987). Cyb2p-like enzymes are not found in *C. elegans* or in mammals. We hypothesized that Cyb2p would oxidize the lactate that accumulates in many cases of mitochondrial dysfunction and contribute to the proton motive force by providing reduced cytochrome *c* for complex IV. When coupled with an endogenous NADH-linked cytosolic lactate dehydrogenase, Cyb2p activity will provide a new pathway for NADH oxidation by the mitochondrial respiratory chain (MRC). Thus, *CYB2* expression should counteract the redox imbalance and lactic acidosis resulting from mitochondrial dysfunction.

Complex I-deficient strains of *C. elegans* expressing *CYB2* demonstrate significantly increased reproductive capabilities, respiration rates, ATP levels, and longer lifespans.

Most importantly, lactate concentrations and the molar ratio of lactate to pyruvate are also significantly improved by Cyb2p. Our results provide the first example of a stable gene therapy strategy with significant benefits in a complex I-deficient animal model system. Our data emphasize the importance of redox imbalance and lactic acidosis in determining the severity of mitochondrial dysfunction.

4.2 Materials and Methods

4.2.1 Strains

Worms were cultured as described (Lewis and Fleming, 1995). We used the following *C. elegans* strains: N2 (Bristol) wild-type; LB25, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx25*[p016bA352V]; LB27, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx27*[p016bA443F]. *uaEx25* and *uaEx27* are extrachromosomal arrays generated by microparticle bombardment that encode *unc-119(+)* for selection and the *nuo-1(A352V)* or the *nuo-1(A443F)* point mutations, respectively (Grad and Lemire, 2004).

4.2.2 Plasmid Constructs

For construction of the *Plet-858*::*CYB2* expression plasmid, a 1.8-kb fragment of DNA encoding the *CYB2* open reading frame was amplified by PCR from *S. cerevisiae* genomic DNA. The oligonucleotides encoded restriction enzyme sites (underlined; forward, *SstI*; reverse, *SalI*) to facilitate cloning into the *C. elegans* expression vector pEXPlet-858 (Ndegwa and Lemire, 2004): forward, 5’-CTGATGTCGACGTCGCTAATACAGTTCCC-3’; reverse, 5’-TAGCTGAGCTCGGCTATAATCATGCATCCTC-3’. pEXPlet-858 is a Gateway Cloning Technology-compatible (Invitrogen Canada, Burlington, ON) vector that contains a 3.5-kb fragment encoding the *let-858* promoter. Construction of the *Pnuo-1::CYB2* expression vector was by Gateway-mediated recombination, placing the *CYB2* gene downstream of a 0.6-kb fragment encoding the *nuo-1* promoter region. All sequences amplified by PCR were confirmed by DNA sequencing.

4.2.3 Generation of Transgenic *C. elegans*

CYB2 expression constructs (17 ng/μl) were co-injected into the syncytial gonads of young adult N2, LB25, and LB27 hermaphrodites with pDP#SU006 (170 ng/μl), a reporter plasmid that carries the gene for the green fluorescent protein (GFP) under the control of the *F25B3.3* promoter (Mello and Fire, 1995). *F25B3.3* encodes the *C. elegans* orthologue of the Ca^{2+}-regulated *ras* nucleotide exchange factor CalDAG-GEFII/RasGRP, which is ubiquitously expressed in the nervous system (Altun-Gultekin *et al.*, 2001). The presence of the transgene was monitored by neuronal-specific GFP expression. Stably-transmitting lines were isolated and the presence of the *CYB2* transgene confirmed by PCR analysis. A minimum of three independent transgenic lines were generated for each strain and *CYB2* construct.

4.2.4 Phenotypic Analyses

Lifespan measurement and brood size determination were performed as described (Grad and Lemire, 2004). For the analysis of animal morphology, adult animals were mounted on 2% agarose pads and observed under a Zeiss Axioskop-2 research microscope equipped with Nomarski and fluorescence optics and a SPOT-2 digital camera (Carl Zeiss Canada, Ltd., Calgary, Canada).

4.2.5 Electrophoresis and Western Blot Analysis

To detect Cyb2p expression, trichloroacetic acid (TCA)-precipitated nematode protein was solubilized in gel loading buffer, resolved by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), and transferred electrophoretically to nitrocellulose membrane. A rabbit polyclonal antiserum raised against *S. cerevisiae* Cyb2p (Dibrov *et al.*, 1998) followed by peroxidase-labeled goat anti-rabbit secondary antibody allowed detection by Enhanced Chemiluminescence (Amersham Biosciences, Buckingham, UK). Blots were stripped and re-probed with a rabbit polyclonal antiserum raised against the *S. cerevisiae* Atp2p (Dibrov *et al.*, 1998; Tsang *et al.*, 2001).

4.2.6 Polarographic Analyses

Oxygen consumption rates were measured using a Strathkelvin 1302 oxygen electrode with an MT200 Mitocell respiration chamber (Strathkelvin Instruments Ltd., Glasgow, UK). For whole-animal respiration rates, synchronized late L4 to early adult worms were washed extensively with M9 buffer to remove bacteria and resuspended to an approximate concentration of 10,000 animals/ml. 60 µl aliquots of resuspended worms were introduced into the Mitocell chamber, which was maintained at 22 °C, and oxygen consumption was measured for at least 10 minutes. Mitochondria-dependent oxygen consumption was terminated by the addition of 1 µl 1 M KCN. The slopes of the linear portions of the plots were used to calculate rates. Samples were carefully recovered from the chamber and the worms lysed with 1.85 M NaOH, 7.4% β-mercaptoethanol. Total protein was recovered by TCA precipitation and solubilized in 50 µl of 5% SDS, 62.5 mM Tris HCl, pH 6.8 for quantification by the BioRad D_C Protein Assay (BioRad Laboratories, Hercules, CA). A minimum of four replicates were performed for each strain.

Mitochondria were isolated from *C. elegans* as described (Grad and Lemire, 2004). L-lactate dependent respiration was measured in isolated mitochondria that had been diluted 10-fold in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, and disrupted by sonication in an ice-water bath for 2-3 min in a Branson 1200 bath sonicator (Branson Ultrasonics Corp., Danbury, CT). To 20 µg mitochondrial protein, rotenone, antimycin A, and L-lactate were added to 100 nM, 75 nM, and 10 mM, respectively and the volume adjusted to 60 µl with 0.1 M HEPES, pH 7.4. Respiration rates were recorded for 2-4 min before terminating the experiment with the addition of 1 µl 1 M KCN. Respiration rates obtained in the absence of L-lactate were measured and subtracted to obtain the L-lactate stimulated rates. Five replicates for each strain and construct were performed to calculate average rates.

4.2.7 Enzyme Assays

Enzyme assays were performed on an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge UK). Rotenone-sensitive NADH-decylubiquinone oxidoreductase activity (complex I) and KCN-sensitive cytochrome *c* oxidase activities were determined as described (Grad and Lemire, 2004). L-lactate-cytochrome *c* oxidoreductase assays were performed at 30 °C using 50 µg disrupted mitochondria as for the succinate-cytochrome *c* oxidoreductase assays, except 10 mM lactate was used as substrate, the inhibitor malonate was omitted, and activity in the absence of lactate was subtracted (Grad and Lemire, 2004). Reported activities represent the mean of at least five determinations.

4.2.8 L-lactate Dehydrogenase Histochemistry

Permeabilization and fixation of worms were performed as described (Xie *et al.*, 1995). An L-lactate dehydrogenase staining protocol was adapted for *C. elegans* (Owen *et al.*, 1982). Briefly, synchronized, fixed. and permeabilized early adult animals were incubated in 1 ml of 5 mM potassium phosphate, pH 7.4, 0.4 mM phenazine methosulfate, 0.5 mM tetranitroblue tetrazolium, and 0.05 M sodium L-lactate for 2 h at 37 °C in the dark with constant rotation. Control samples were incubated in the absence of lactate. Stained worms were centrifuged for 5 min at 360 x *g* and washed three times for 5 min in phosphate buffer before mounting for photography.

4.2.9 Measurement of Metabolite Concentrations

For the determination of lactate and pyruvate concentrations, worms were cultured in liquid medium, harvested, extensively washed, and resuspended in 27 ml water at a concentration of approximately 20 mg protein/ml. Protein-free extracts were obtained by TCA precipitation (Grad and Lemire, 2004). To determine total ATP content, synchronized late L4 to early adult animals were washed repeatedly in S Basal buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH 6.0), resuspended in a final volume of 100 µl, and frozen at –80 °C. The frozen worms were immediately immersed in a boiling water bath for 15 minutes, cooled, and centrifuged to pellet lysed nematodes. The supernatant was removed to a fresh tube and serially diluted. ATP content was determined using the ATP Bioluminescence Assay Kit CLS II (Roche Molecular Biochemicals, Mannheim, Germany) with a Berthold Lumat LB 9501 luminometer (Berthold Technologies (USA), Oakridge, TN). The values reported are means of at least three independent determinations.

4.2.10 Oxidative Stress Assays

A minimum 50 L1 larvae were placed onto seeded NGM plates and incubated at 22 °C in an atmosphere of 100% oxygen or on plates containing 0.2 mM paraquat (Grad and Lemire, 2004). Survival, measured as the fraction of L1 larvae that developed into adults, was scored after 5 days. A minimum of ten trials were performed for each strain and construct.

4.3 Results

4.3.1 Generation of Transgenic C. elegans Strains Expressing CYB2

The *S. cerevisiae* gene *CYB2* was cloned into *C .elegans* expression vectors under the control of the gene *let-858* or the *nuo-1* promoters. The *let-858* gene is a member of the lethal gene class and encodes nucampholin, a ubiquitously expressed protein required for early embryogenesis and tissue differentiation (Kelly *et al.*, 1997). The *nuo-1* gene encodes a known mitochondrial protein, the 51-kDa subunit of complex I (Tsang *et al.*, 2001). Both promoters should result in the ubiquitous expression of *CYB2*. *Plet-858::CYB2* and *Pnuo-1::CYB2* were co-injected with a reporter plasmid that expresses GFP in the nervous system into the wild-type strain N2 and into the complex I-deficient strains LB25 and LB27 (Grad and Lemire, 2004). LB25 carries on the *uaEx25* extrachromosomal array, a mutated version of *nuo-1* encoding an A352V substitution. Similarly, LB27 expresses a version of *nuo-1* with an A443F substitution. LB25 and LB27 are both homozygous for the *nuo-1(ua1)* allele, a lethal deletion believed to be a null mutation (Tsang *et al.*, 2001). LB25 and LB27 were chosen because their overall fitness is markedly impaired and their phenotypes include features typical of mitochondrial diseases such as riboflavin- and dichloroacetate-responsive lactic acidosis. In the strains we chose to investigate in detail, the *Plet-858::CYB2* and *Pnuo-1::CYB2* transgenes were transmitted to progeny at rates of 70 ± 8% and 64 ± 9%, respectively, for the N2 strains, 51 ± 7% and 62 ± 8%, respectively, for the LB25 strains, and 62 ± 12% and 51 ± 9%, respectively, for the LB27 strains.

4.3.2 Characterization of CYB2 Transgenic Strains

CYB2 expression is strongly beneficial to overall animal health and fertility. We noted significant increases in the average brood size of 44-54% in LB25 and 92-123% in LB27 strains expressing *CYB2* compared to their parental mutant strains (Figure 4.1A, B). Similarly, the average lifespans of LB25 and LB27 are increased 25-28% and 24-31%, respectively, with *CYB2* expression. The *CYB2* constructs had no significant effects on N2 strains.

LB25 and LB27 nematodes display gross abnormalities in gonad morphology and muscle tissue integrity (Grad and Lemire, 2004). Mutant adults often have shortened or malformed gonad arms with disorganized syncytia that appear shriveled, leaving space between somatic gonad tissue and the body wall. LB25 and LB27 strains carrying *CYB2* transgenes displayed significantly lower frequencies of abnormal gonad morphology and fewer signs of tissue degeneration in 1-day-old adult hermaphrodites. Many young adult mutants have large vacuole-like structures throughout the body, beneath the cuticle, suggesting premature degeneration of body wall muscle tissue (Figure 4.2). Approximately 60% and 67% of one-day old adult LB25 and LB27 hermaphrodites, respectively, display prominent signs of tissue degeneration in the head region. Only 38-40% and 39-41% of similarly aged animals do so when carrying the *CYB2* transgene. Abnormal gonad morphology is present in about 69% and 78% of 1-day-old adult LB25 and LB27 hermaphrodites, respectively and decreases to 32-34% and 36-44%, respectively, with *CYB2* expression (Figure 4.3). The *CYB2* constructs had no significant effects on the gonad morphology of N2 strains.

4.3.3 Cyb2p is Expressed and Functionally Active

Cyb2p expression in the transgenic *C. elegans* strains was confirmed by Western blot analysis using a polyclonal antiserum against the *S. cerevisiae* Cyb2p (Dibrov *et al.*, 1998). Cyb2p is synthesized as a protein precursor of 591 amino acids (65.5 kDa) that includes an 80 residue bipartite N-terminal mitochondrial presequence (Neupert, 1997). The Cyb2p presequence is removed by proteolysis upon successful import to the mitochondrial matrix and subsequent sorting to the intermembrane space, resulting in the production of mature Cyb2p consisting of 511 residues (56.6 kDa). A Cyb2p-specific signal with an apparent molecular weight of just under 58-kDa, similar in size to native Cyb2p in yeast mitochondria, was detected only in *C. elegans* samples from Cyb2p-expressing strains (Figure 4.4). We believe this species corresponds to the mature form of Cyb2p, indicating its successful import, sorting, and processing into nematode mitochondria. We did not detect higher molecular weight forms that might correspond to the precursor. As a loading control, we monitored the steady-state protein levels of ATP-2, the β-subunit of the ATP synthase, which do not vary in the complex I-deficient mutants (Grad and Lemire, 2004).

Our data strongly suggest that Cyb2p is functional in nematode mitochondria and contributes bioenergetically. We measured the L-lactate-dependent cytochrome *c* reductase activity in isolated worm mitochondria. Significant activity was only detectable in mitochondria from Cyb2p-expressing strains (Table 4.1). Cyb2p expression did not affect the activities of complexes I or IV, which are compromised in LB25 and LB27 (Grad and Lemire, 2004). Polarographic analysis of oxygen consumption rates revealed significantly higher L-lactate-dependent respiration in isolated mitochondria of

Cyb2p-expressing strains (Figure 4.5) L-lactate dehydrogenase activity, detected as the black precipitate formed upon the phenazine methosulfate-mediated reduction of tetranitroblue tetrazolium, was also assessed histochemically in transgenic strains (Figure 4.6). L-lactate dehydrogenase activity appears most abundant in the anterior body wall muscles surrounding the pharyngeal bulbs of young adult hermaphrodites. The *nuo-1* promoter appeared to result in lower activity in the anterior regions, but more activity in muscle tissue throughout the body (data not shown). Finally, we measured whole-animal respiration by monitoring oxygen consumption in live nematodes. LB25 and LB27 are characterized by significantly decreased respiration rates that are approximately half of wild-type (Grad and Lemire, 2004). Oxygen consumption rates increased 32-41% and 54-58% in LB25 and LB27, respectively, when Cyb2p was expressed (Figure 4.7A). Respiration in Cyb2p-expressing N2 animals was not affected.

4.3.4 Cyb2p Contributes to Energy Generation

CYB2 expression improves animal health and increases respiration rates and thus likely contributes to energy production in transgenic strains. We determined ATP levels from extracts of synchronized, late L4-young adult hermaphrodites. LB25 and LB27 have severely depressed total ATP concentrations that are 20-30% of wild-type (Figure 4.7B). All mutant strains expressing *CYB2* demonstrated significant elevations in ATP concentrations of approximately 2-3-fold. These levels, however, remain below wild-type levels, suggesting that Cyb2p-mediated respiration cannot fully compensate for the diminished complex I function. Taken together, our data demonstrate that *CYB2* expression leads to increased respiration rates that contribute to the increased production of ATP through oxidative phosphorylation.

4.3.5 CYB2-Expression Moderates Lactic Acidosis

A key indicator of mitochondrial dysfunction is metabolic acidosis resulting from a diminished ability to oxidize NADH (Chinnery and Turnbull, 2001; DiMauro *et al.*, 2004; Munnich *et al.*, 1996; Scaglia *et al.*, 2004). LB25 and LB27 have significantly increased molar ratios of lactate to pyruvate, reflecting the metabolic imbalance in the metabolism of NADH (Grad and Lemire, 2004). Protein-free extracts of *CYB2*-expressing strains contain significantly lower concentrations of lactate (23-35% in LB25 and 28-44% in LB27), and correspondingly lower lactate to pyruvate ratios (Table 4.2). The lactate concentrations in the transgenic LB25 and LB27 strains approach those seen in wild-type animals, although the molar ratios of lactate to pyruvate do not fully return to wild-type levels. Pyruvate concentrations are relatively unchanged in all LB25 strains but are increased in LB27 strains, suggesting the A352V and A443F mutations may differ in the metabolic details of pathogenesis. No significant changes in lactate or pyruvate levels were observed in transgenic N2 animals. Our data strongly suggest that *CYB2* expression can provide an alternate pathway for oxidizing lactate and NADH and thus partially correct the redox imbalance seen in the complex I-deficient mutants.

4.3.6 CYB2-Expressing C. elegans have Increased Hypersensitivity to Oxidative Stress

Mitochondrial dysfunction can result in accelerated, pathogenic rates of reactive oxygen species generation that lead to protein and nucleic acid damage and hypersensitivity to conditions of oxidative stress (Senoo-Matsuda *et al.*, 2001). LB25 and LB27 are hypersensitive to oxidative stress caused by hyperoxia or exposure to paraquat, a known generator of superoxide radicals (Grad and Lemire, 2004). The hypersensitivity could be partially alleviated pharmacologically. Surprisingly, transgenic strains expressing *CYB2* showed increased sensitivity to oxidative stress (Figure 4.8A, B). Transgenic N2 strains developed significant hypersensitivity to paraquat (Figure 4.8B). These data suggest that *CYB2* expression may exacerbate reactive oxygen species generation in *C. elegans* mitochondria under conditions of oxidative stress.

4.4 Discussion

Elucidating the relationships between genotype and phenotype is a major challenge in the field of mitochondrial medicine. MRC dysfunction can result in at least four mechanisms of pathology: 1) abnormal cellular redox states, 2) the impairment of numerous metabolic pathways that require MRC function, 3) the generation of reactive oxygen species, and 4) decreased ATP formation (Munnich and Rustin, 2001). The complex I-deficient strains of *C. elegans* we have generated provide evidence that all four of these mechanisms contribute to the mutant phenotypes. Diagnosis and treatment of mitochondrial disease will require a more thorough understanding of these mechanisms of pathology and how they combine to produce the observed clinical presentation

The pathogenic effects of MRC deficiency are complex and cannot be simply explained by just a lack of cellular energy. Our results indicate that an abnormal redox state is also a major determinant of the phenotype of complex I-deficient nematodes. The *nuo-1* mutations we studied affect the 51-kDa active site subunit of complex I and impair NADH-dependent mitochondrial respiration, leading to elevated NADH/NAD$^+$ ratios and the NADH-dependent reduction of pyruvate to lactate. Mutant fitness was most responsive to pharmacological agents that stimulate complex I activity and reduce the levels of NADH, or stimulate pyruvate dehydrogenase activity, which should reduce the levels of pyruvate (Grad and Lemire, 2004). The severity of the mutant phenotypes is correlated to the level of lactic acidosis. These observations underlie the rationale for developing a gene therapy strategy that provides an alternative pathway for the metabolism of lactate.

In this study, we demonstrate that expression of the yeast Cyb2p, which encodes a lactate-cytochrome *c* oxidoreductase localized to the mitochondrial intermembrane space, introduces a new pathway for lactate and NADH oxidation. This new metabolic ability results in significant improvements in animal fitness as measured by fertility and lifespan. *CYB2*-expression often restored the highly organized developmental processes needed to produce the adult germline and somatic gonad. It also resulted in significantly fewer signs of premature body wall muscle tissue degeneration, suggesting that addressing metabolic imbalances in disease can result in a broad range of beneficial effects.

Import, processing, and targeting of the proteins to mitochondria are highly conserved amongst widely-divergent eukaryotes (Glerum and Tzagoloff, 1994; Hashimoto *et al.*, 1999; Wang *et al.*, 2001; Rodríguez-Aguilera *et al.*, 2003). Several lines of evidence suggest that the heterologous expression of the yeast *CYB2* produced functional Cyb2p in nematode mitochondria. We immunologically detected Cyb2p, which co-migrated with mature Cyb2p from yeast mitochondria, indicating it was properly processed. L-lactate dependent cytochrome *c* reductase activity was only present in mitochondria of *CYB2*-expressing strains. Cyb2p activity was also detected *in situ* via histochemical staining. Finally, we demonstrated that *CYB2* expression resulted in lactate-dependent respiration in isolated mitochondria and in increased total respiration in live animals.

CYB2 expression was driven by the *let-858* and the *nuo-1* promoters, both believed to drive ubiquitous expression. The Cyb2p expression patterns, as judged histochemically, were largely similar between the two promoter constructs. In addition, *CYB2* expression with both promoters produced similar beneficial effects in the complex I-deficient nematodes.

Cyb2p expression may mediate its beneficial effects in at least two ways. First, lactate oxidation is coupled to the reduction of cytochrome *c* and complex IV activity. Complex IV pumps protons and contributes to the electrochemical proton gradient that drives ATP synthesis. We showed that Cyb2p expression results in the elevation of ATP levels in transgenic strains. This is not surprising given that mitochondria are the major site of ATP synthesis. Second, Cyb2p functions as an L-lactate dehydrogenase, which when coupled with an endogenous NADH-linked cytosolic lactate dehydrogenase, reduces the cellular levels of lactate and NADH, thus correcting the redox imbalance (Figure 4.9). We demonstrated significantly decreased lactate concentrations and decreased molar ratios of lactate to pyruvate in *CYB2*-expressing mutants. Correcting the redox imbalance may also influence the activities of other pathways such as glycolysis, the pentose phosphate pathway, the citric acid cycle, and fatty acid oxidation; pathways that require NAD^+ or $NADP^+$ for their operation. Lactic acidosis reduces the intracellular pH and restoring the pH may also correct metabolism more globally. The increased live animal respiration rates we observed in *CYB2*-expressing strains support our contention that overall metabolism is stimulated by the correction in redox imbalance.

CYB2 expression does not correct the MRC defects: the activities of complexes I and IV are not increased in the transgenic strains (Table 4.1). An unexplained consequence of the complex I mutations is the impaired activity and assembly of complex IV (Grad and Lemire, 2004). The assembly of the mtDNA-encoded subunits of complex IV is dependent on the membrane potential across the mitochondrial inner membrane (Hell *et al.*, 2001). If complex IV assembly is impaired by a diminished membrane potential in the *nuo-1* mutants, we would have predicted that Cyb2p expression would at least partially restore its assembly since Cyb2p stimulates both respiration and ATP generation.

Chronic redox imbalance may lead to deleterious effects on gene expression, which may contribute to the progressive nature of mitochondrial diseases. For example, the Sir2p family of proteins are NAD$^+$-dependent histone deacetylases that affect gene silencing (Guarente, 2000). The mammalian homologue of Sir2p, SIRT1, deacetylates not only histones, but also key transcription factors (Bordone and Guarente, 2005). Furthermore, SIRT3, another homologue of Sir2p, can activate mitochondrial function and is localized to the mitochondrial inner membrane (Shi *et al.*, 2005). Addressing redox imbalance may provide both short- and long-term benefits in the treatment of mitochondrial disease.

Surprisingly, *CYB2* expression increased the hypersensitivity of complex I mutants to oxidative stress. That complex I mutations elicit sensitivity to oxidative stress is not surprising because complex I is a major site of reactive oxygen species production in the MRC (Raha and Robinson, 2000; Raha and Robinson, 2001). The pharmacological agents that addressed lactic acidosis also provided protection from oxidative stress, suggesting a mechanistic connection between oxidative stress and redox imbalance (Grad and Lemire, 2004). Although Cyb2p clearly ameliorates the redox imbalance, it also increases sensitivity. This phenomenon is independent of complex I dysfunction since wild-type nematodes expressing *CYB2* were also rendered hypersensitive to paraquat. The Cyb2p-dependent production of reactive oxygen species must be mechanistically different during exposure to paraquat or to hyperoxia, since only the former affects the survival of wild-type worms. One possible explanation is that Cyb2p is itself a site of reactive oxygen species production, possibly through its flavin or heme cofactors. Alternatively, the increased flow of electrons in the Cyb2p-containing mitochondria or the increased membrane potential as a result of the increased electron flow may stimulate the production of reactive oxygen species at other sites in the MRC, such as complex III. Further study will be needed to elucidate the exact mechanisms by which Cyb2p leads to sensitivity to oxidative stress.

In conclusion, we have demonstrated the successful use of a novel gene therapy strategy, one that introduces an alternate metabolic pathway to mitigate the effects of mitochondrial dysfunction. The yeast *CYB2* gene targets the redox imbalance and lactic acidosis caused by complex I dysfunction, resulting in significant improvements in animal fitness. Our data strongly suggest that the disruption of cellular metabolism associated with redox imbalance and ATP generation are central mechanisms to the pathology of mitochondrial diseases.

Table 4.1 Mitochondrial respiratory enzyme assays.

Strain	Complex I[a]	Complex IV[b]	Cyb2p[c]
N2	65.8 ± 3.5[d]	28.4 ±1.5	N.D.[e]
N2 + *Pnuo-1::CYB2*	64.0 ± 3.8	28.6 ± 1.6	3.9 ± 1.4
N2 + *Plet-858::CYB2*	61.7 ± 2.1	27.4 ± 1.9	3.8 ± 1.1
LB25	26.4 ± 2.9	15.5 ± 1.9	N.D.
LB25 + *Pnuo-1::CYB2*	25.8 ± 2.9	14.8 ± 0.6	4.2 ± 1.6
LB25 + *Plet-858::CYB2*	28.8 ± 2.3	16.0 ± 1.0	6.8 ± 2.7
LB27	26.1 ± 2.9	14.9 ± 1.2	N.D.
LB27 + *Pnuo-1::CYB2*	29.4 ± 2.3	13.9 ± 1.4	7.3 ± 2.2
LB27 + *Plet-858::CYB2*	26.7 ± 5.6	14.6 ± 1.4	8.4 ± 3.0

[a] Complex I activity was measured as the rotenone-sensitive NADH-decylubiquinone reductase activity. The values reported are nmol NADH min^{-1} mg protein^{-1}. [b] Complex IV activity was measured as the initial rate of cyanide-sensitive cytochrome *c* oxidase activity. The values reported are nmol cytochrome *c* min^{-1} mg protein^{-1}. [c] Cyb2p activity was measured as L-lactate-dependent cytochrome *c* reductase activity. The values reported are nmol cytochrome *c* min^{-1} mg protein^{-1}. [d] The values reported are means ± standard deviation of a minimum of four measurements. [e] N.D., not detected.

Table 4.2 Lactate and pyruvate concentrations of transgenic animals.

Strain	Lactate (mM)	Pyruvate (mM)	L:P
N2	2.43 ± 0.51[a]	0.148 ± 0.026	16.4 ± 4.5
N2 + *Pnuo-1::CYB2*	1.95 ± 0.18	0.117 ± 0.010	16.7 ± 2.1
N2 + *Plet-858::CYB2*	1.92 ± 0.12	0.114 ± 0.011	16.8 ± 1.9
LB25	3.06 ± 0.32	0.063 ± 0.007	48.6 ± 7.4
LB25 + *Pnuo-1::CYB2*	2.36 ± 0.12*	0.068 ± 0.003	34.7 ± 2.5*
LB25 + *Plet-858::CYB2*	2.00 ± 0.17*	0.062 ± 0.004	32.3 ± 3.4*
LB27	4.23 ± 0.75	0.052 ± 0.004	81.3 ± 15.6
LB27 + *Pnuo-1::CYB2*	3.03 ± 0.13	0.076 ± 0.007*	39.9 ± 4.0*
LB27 + *Plet-858::CYB2*	2.37 ± 0.53*	0.063 ± 0.007	37.6 ± 9.3*

[a] The values reported are the means ± standard deviation of a minimum of four determinations. *$P < 0.05$, compared to corresponding parental strain using a two-sample *t*-test.

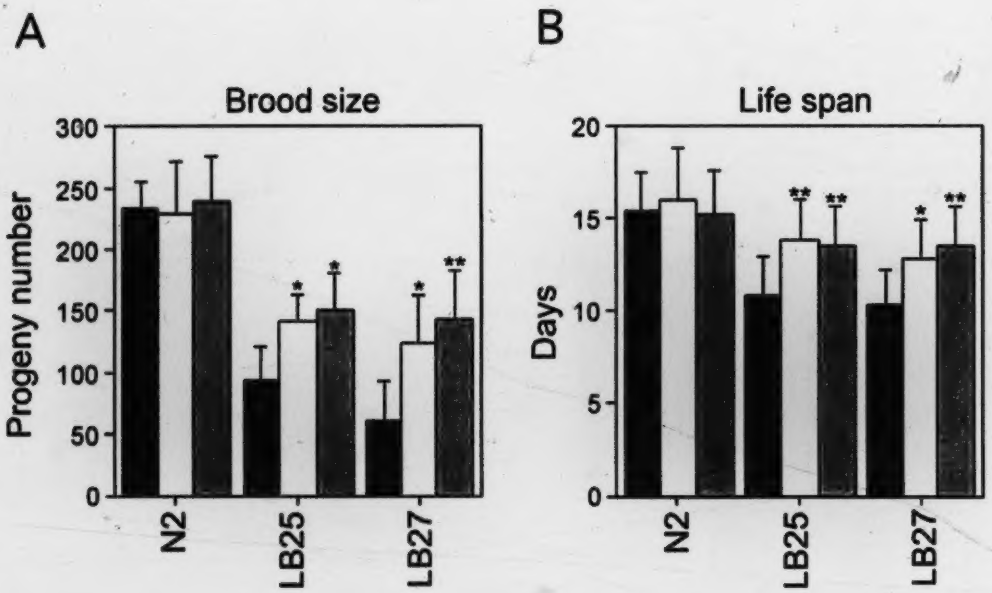

Figure 4.1 Effects of *CYB2* expression on brood sizes and life spans. **(A)** Transgenic animals were allowed to lay eggs at 20 °C and all progeny were counted. The values reported are averages of at least 18 broods counted. **(B)** Transgenic animals cultured at 20 °C were monitored daily and scored as dead when they no longer responded to gentle prodding on the head. The values reported are averages of a minimum of 24 animals. Black bars represent no *CYB2* transgene; white bars represent presence of *Pnuo-1*::*CYB2* transgene; gray bars represent presence of *Plet-858*::*CYB2* transgene. All *P*-values (*, $P<0.05$; **, $P<0.01$) are derived from a two-sample *t*-test when comparing with the corresponding strain without *CYB2*.

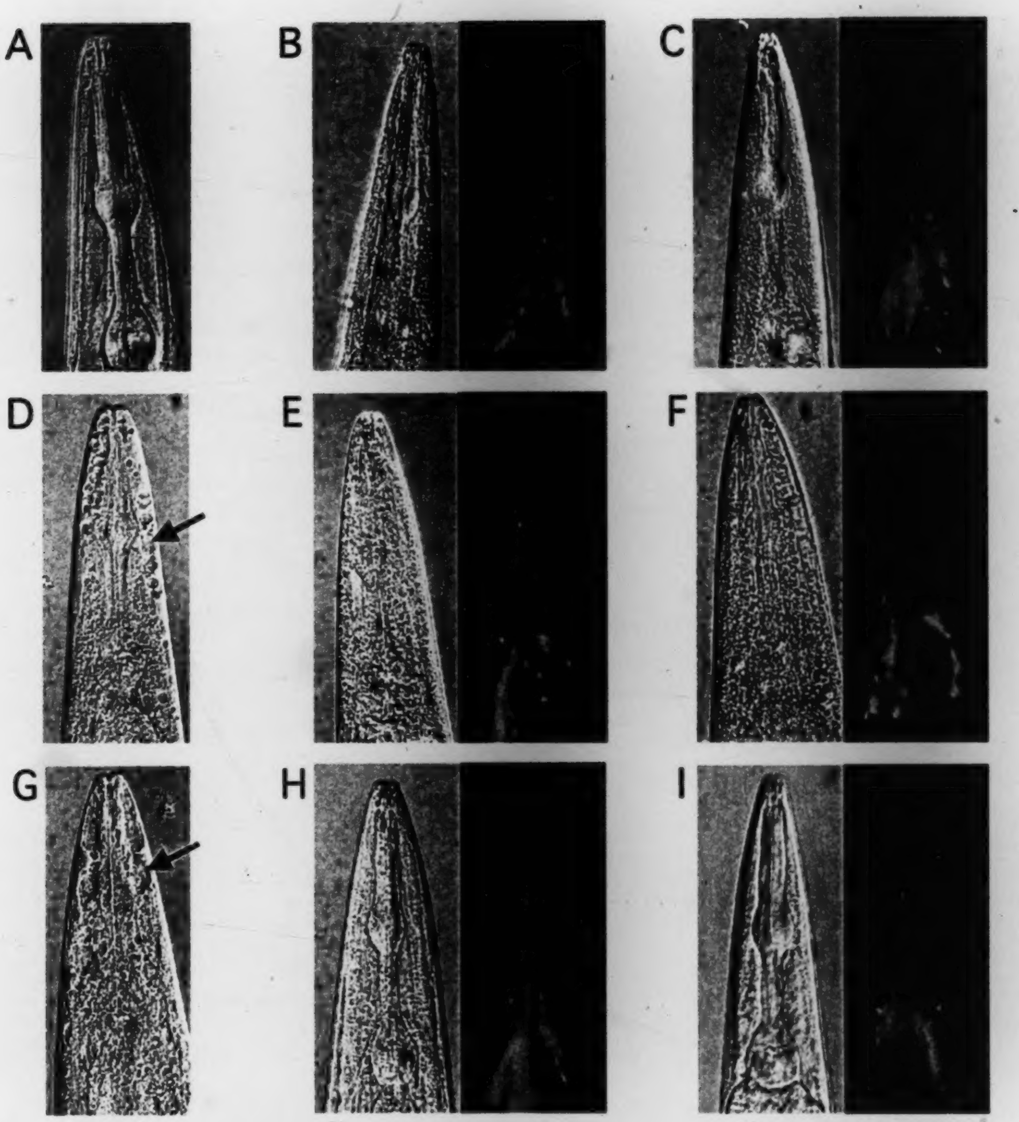

Figure 4.2 Nomarski photographs of head regions of 1 day-old adults. (A) N2; (B) N2 with *Pnuo-1*::*CYB2*; (C) N2 with *Plet-858*::*CYB2*; D) LB25; E) LB25 with *Pnuo-1*::*CYB2*; (F) LB25 with *Plet-858*::*CYB2*; (G) LB27; (H) LB27 with *Pnuo-1*::*CYB2*; (I) LB27 with *Plet-858*::*CYB2*. The arrows indicate examples of vacuole-like structures. For strains carrying the *CYB2* transgene, the corresponding UV fluorescence image is shown indicating the presence of the GFP marker associated with the transgenic array.

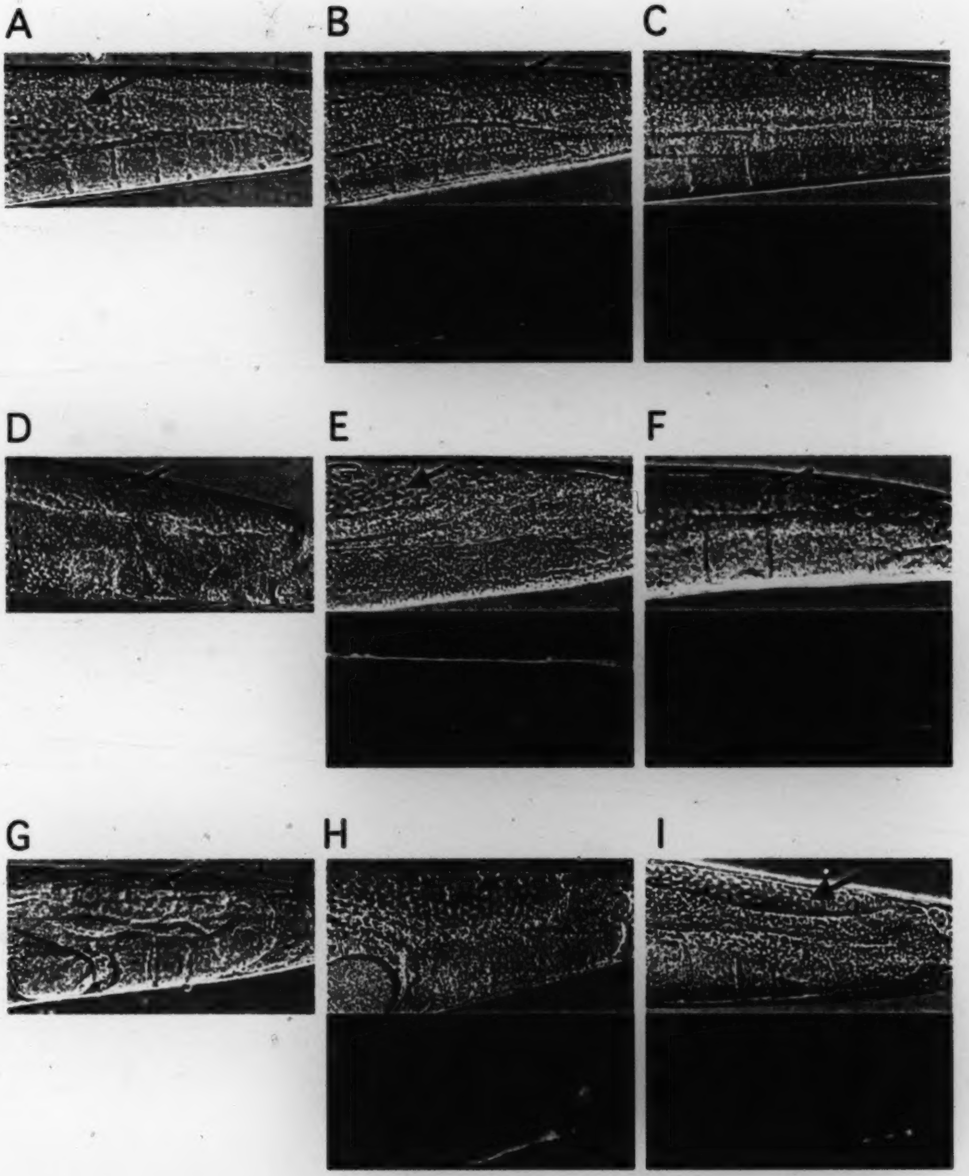

Figure 4.3 Nomarski photographs of posterior gonad arms of 1 day-old adults. (A) N2; **(B)** N2 with *Pnuo-1*::*CYB2*; **(C)** N2 with *Plet-858*::*CYB2*; **(D)** LB25; **(E)** LB25 with *Pnuo-1*::*CYB2*; **(F)** LB25 with *Plet-858*::*CYB2*; **(G)** LB27; **(H)** LB27 with *Pnuo-1*::*CYB2*; **(I)** LB27 with *Plet-858*::*CYB2*. The arrows indicate the syncytial germline nuclei. For strains carrying the *CYB2* transgene, the corresponding UV fluorescence image is shown indicating the presence of the GFP marker associated with the transgenic array.

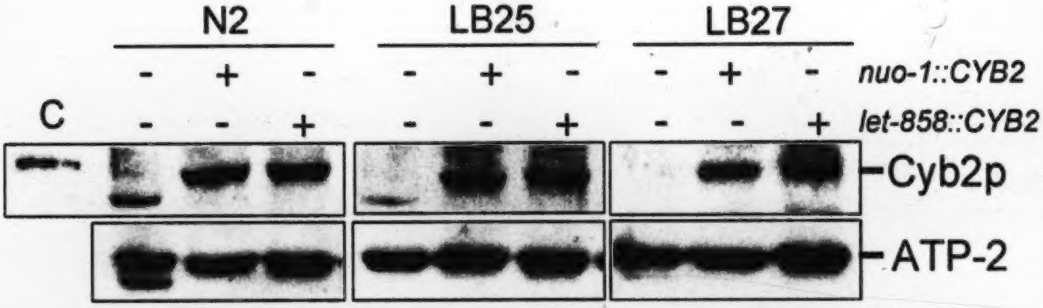
N2
LB25
LB27
- + - - + - - + -
nuo-1::CYB2
C
- - + - - + - - +
let-858::CYB2
Cyb2p
ATP-2

Figure 4.4 Detection of Cyb2p in whole-nematode lysates by Western blot analysis. 100 μg nematode protein lysate were loaded per lane. The blot was stripped and re-probed with antiserum against ATP-2. C, yeast mitochondrial protein control (0.5 μg).

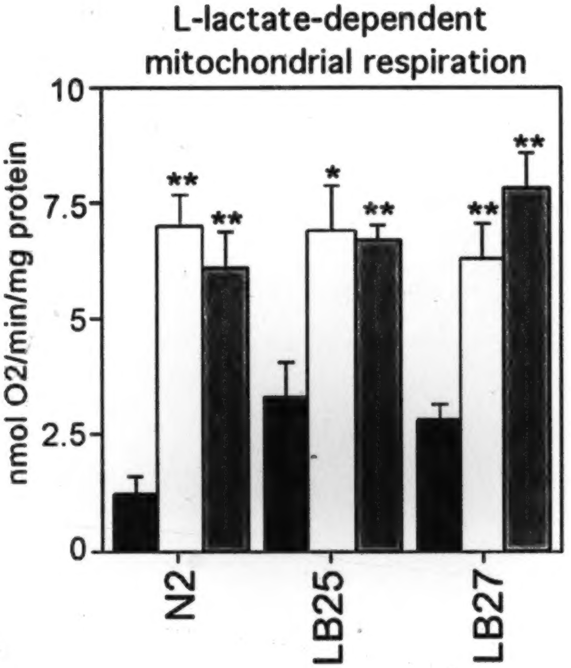

Figure 4.5 L-lactate-dependent respiration in isolated mitochondria. The values reported are the means of at least five measurements. Black bars represent no *CYB2* transgene; white bars represent presence of *Pnuo-1*::*CYB2* transgene; gray bars represent presence of *Plet-858*::*CYB2* transgene. All *P*-values (* $P<0.05$; ** $P<0.01$) are derived from a two-sample *t*-test when comparing with the corresponding strain without *CYB2*.

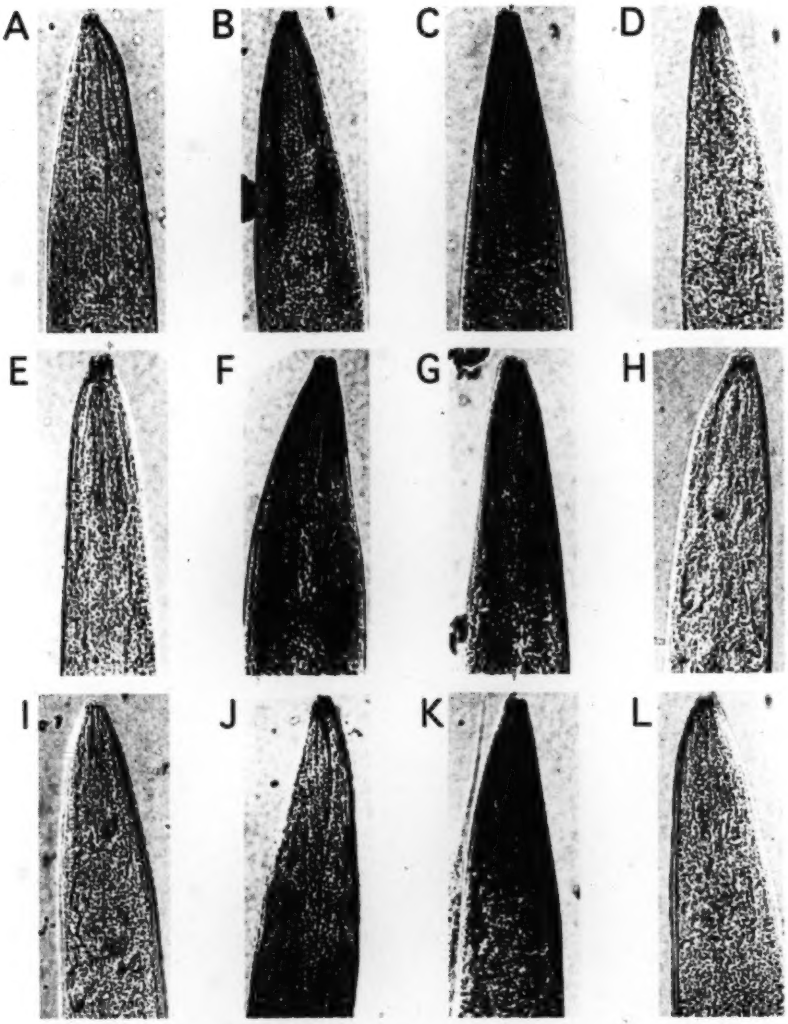

Figure 4.6 Histochemical detection of L-lactate dehydrogenase activity. Nomarski photographs of head regions from 1 day-old adult hermaphrodites stained in the presence of 0.05 M sodium L-lactate, except when stated. **(A)** N2; **(B)** N2 with *Pnuo-1*::*CYB2*; **(C)** N2 with *Plet-858*::*CYB2*; **(D)** N2 with *Plet-858*::*CYB2* in the absence of L-lactate; **(E)** LB25; **(F)** LB25 with *Pnuo-1*::*CYB2*; **(G)** LB25 with *Plet-858*::*CYB2*; **(H)** LB25 with *Plet-858*::*CYB2* in the absence of L-lactate; **(I)** LB27; **(J)** LB27 with *Pnuo-1*::*CYB2*; **(K)** LB27 with *Plet-858*::*CYB2*; **(L)** LB27 with *Plet-858*::*CYB2* in the absence of L-lactate.

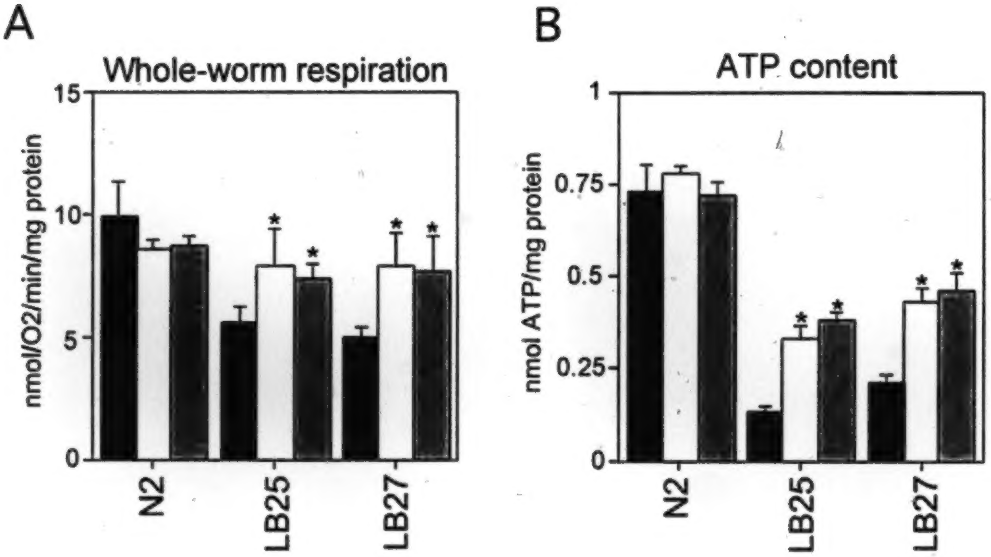

Figure 4.7 Cyb2p improves the overall metabolism of transgenic strains. (A) Oxygen consumption rates of live nematodes. The values reported are the means of five measurements. **(B)** Total ATP contents of transgenic animals. The values reported are the averages of three independent determinations. Black bars represent no *CYB2* transgene; white bars represent presence of *Pnuo-1*::*CYB2* transgene; gray bars represent presence of *Plet-858*::*CYB2* transgene. All *P*-values (* $P<0.05$; ** $P<0.01$) are derived from a two-sample *t*-test when comparing with the corresponding strain without *CYB2*.

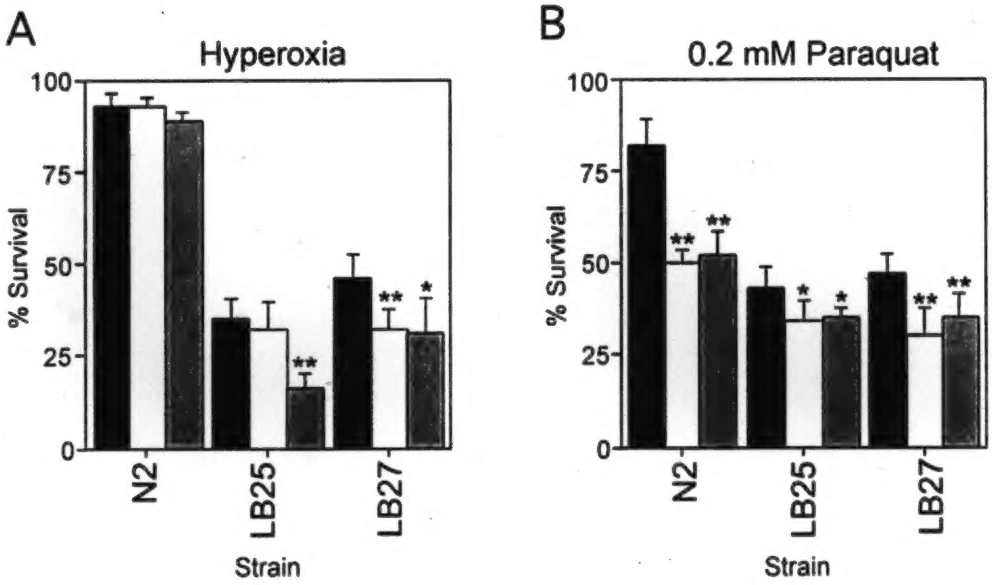

Figure 4.8 The effects of oxidative stress on transgenic strains expressing *CYB2*. Each value is the mean of ten measurements. L1 larvae were placed onto seeded NGM plates and incubated at 22 °C **(A)** in a 100% oxygen atmosphere; **(B)** NGM plates containing 0.2 mM paraquat. Black bars represent no *CYB2* transgene; white bars represent presence of *Pnuo-1*::*CYB2* transgene; gray bars represent presence of *Plet-858*::*CYB2* transgene. All *P*-values (* $P<0.05$; ** $P<0.01$) are derived from a two-sample *t*-test when comparing with the corresponding strain without *CYB2*.

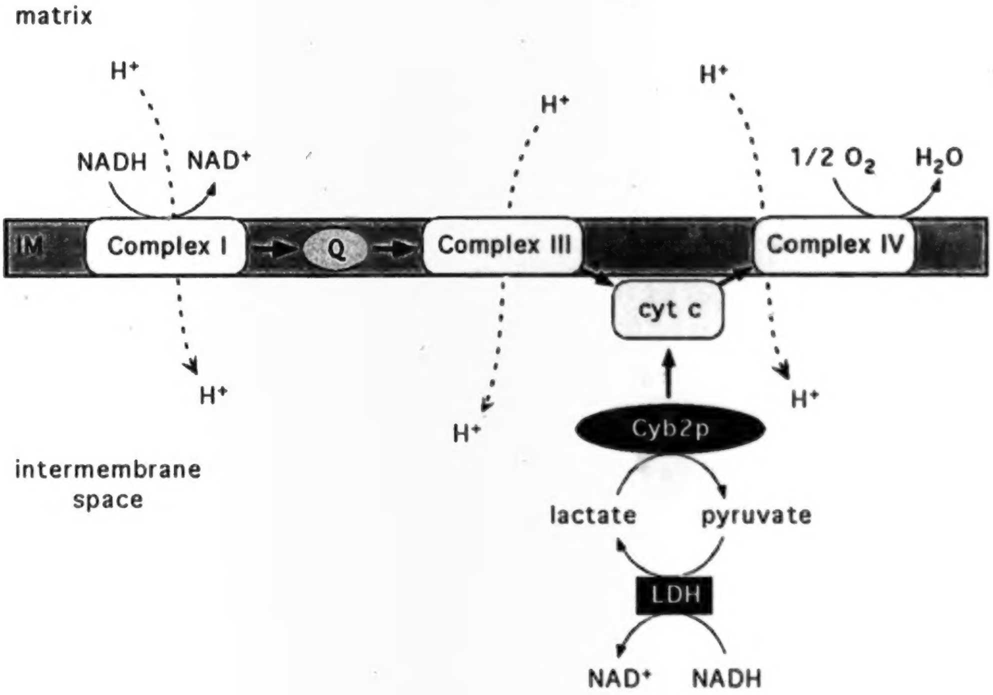
matrix
H+
H+
H+
NADH
NAD+
1/2 O2
H2O
IM
Complex I
Q
Complex III
Complex IV
cyt c
H+
H+
H+
Cyb2p
intermembrane space
lactate
pyruvate
LDH
NAD+
NADH

Figure 4.9 Cyb2p-mediated oxidation of lactate and NADH. Complexes I, III, and IV are integral membrane proteins found in the mitochondrial inner membrane (IM). They translocate protons from the matrix to the intermembrane space. The mitochondrial outer membrane (not shown) is not a permeability barrier; small molecules such as lactate, pyruvate, NAD^+, and NADH can move between the cytosol and the intermembrane space. LDH, an endogenous, cytosolic lactate dehydrogenase; Q, ubiquinone; cyt *c*, cytochrome *c*.

4.5 Bibliography

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Chapter 5

General Discussion and Conclusion

5.1 Conclusion

The aim of this thesis work was to gain insight into the molecular mechanisms of pathogenesis caused by MRC mutations. Although not considered one of the traditional genetic systems for the study of mitochondrial function or even of human disease, we have demonstrated in this collection of studies the success and future potential of the nematode system. Not only do these investigations open the door to further exploration of additional pathogenic MRC mutations but they also serve as a guide for investigating a range of rare or sporadic disorders that are poorly understood. Furthermore, disease models in *C. elegans* provide an excellent testing ground for the systematic development of new therapies and treatments that will expand the range of mitochondrial medicine. In this final section, I will discuss some future directions to pursue relating to the investigations reported in this thesis. I will briefly recount the limitations of the nematode in this field of research. I will also discuss a relatively new diagnostic tool used in the study of mitochondrial disorders, called metabolic profiling. Metabolic profiling is a derivative of a new field of study in the post-genomic era referred to as 'metabolomics'. This discussion will in part be based on my own preliminary nuclear magnetic resonance (NMR) experiments using the nematode. Finally, I will discuss other gene therapy strategies that can be conducted in *C. elegans* to redress the effects of MRC pathogenesis.

5.2 Discussion and Future Directions

5.2.1 Continuing Investigations of nuo-1 Mutants in C. elegans

The complexities of MRC dysfunction, and the rare and sporadic nature of many mitochondrial disorders, demand the use of a convenient and pliable model system for their study. We have developed the nematode *C. elegans* as such an experimental system. As summarized in subsection 1.6.2, the majority of mitochondrial research in *C. elegans* has utilized a forward genetics approach; a mutant phenotype was identified prior to the identification of the gene responsible for it. These studies have provided valuable insight into MRC dysfunction and its effects on a multicellular, multisystemic organism. This insight could never be achieved by using unicellular eukaryotic model systems such as *S. cerevisiae*. However, many *C. elegans* investigations have dealt with situations that are unlikely to occur in humans. These include complete loss-of-function mutations, which would surely be lethal. The studies in this thesis present the first example of an applied investigation that uses a reverse genetic approach to study mitochondrial disorders in the nematode.

The principal focus of this thesis is the modeling of specific missense mutations first identified in the human *NDUFV1* gene using the *C. elegans* orthologue, *nuo-1*. Although three mutations were characterized in the thesis, an additional five independent *NDUFV1* mutations have been reported; three are missense mutations resulting in single amino-acid substitutions: Y204C, C206G and E214K (Bénit *et al.*, 2001). Interestingly, these three mutations are all located within the predicted FMN-binding domain of the protein (Fearnley and Walker, 1992). The obvious next step would be to model these missense mutations. This trio of new mutations is already known to directly affect a cofactor binding site. It would be interesting to determine if all three mutations are equally severe and if they are responsive to riboflavin through comparative examination of all six known NDUFV1 amino-acid substitutions. This would undoubtedly further clarify the pathogenic mechanisms associated with complex I dysfunction and the functional importance of each these residues within the protein.

Has creating a *C. elegans* mitochondrial disease model system taught us anything new regarding the mechanisms of mitochondrial disease? In the broadest of terms, the molecular mechanisms of pathogenesis as observed in our *C. elegans* complex I mutants involve processes that have long been associated with MRC dysfunction and mitochondrial disorders: oxidative stress, premature apoptosis, metabolic imbalance and the misassembly of MRC complexes. However, observing these mechanisms does reinforce the high relevance of the nematode for this kind of research. Our studies have revealed a hierarchy to the pathogenic mechanisms, both in terms of their temporal order and their relative impact on the organism. For example, the studies in Chapter 4 suggest that redox imbalance caused by the inability of complex I to oxidize NADH to NAD^+ is a major cause of the deleterious effects observed in the *nuo-1* mutants. Redox imbalance is a direct result of the catalytic deficiency in complex I and can affect a large number of downstream targets with a consequently large impact on the health of the organism. The effects of the redox imbalance can contribute to the aberrant regulation of metabolic pathways or of gene expression. In addition, metabolic acidosis itself is often the cause of death in patients with complex I mutations (Bénit *et al.*, 2001). These observations suggest that therapies or treatments addressing acidosis and redox imbalance may have a great impact clinically.

The effects of the redox imbalance should be investigated in greater detail. As alluded to in Chapter 4, the NADH/NAD$^+$ ratio is linked to gene expression, most notably through the activity of highly conserved Sir2 family members, which act as NAD$^+$-dependent deacetylases and control gene silencing (Guarente, 2000). An examination of Sir2 family member expression and/or deacetylase activity in the *nuo-1* mutants may reveal the importance of gene regulation in the severity and progression of mitochondrial diseases. One approach would involve the creation of a NAD$^+$-sensitive reporter construct that measures the activity of the *C. elegans sir-2.1* gene in a complex I-deficient background. Alternatively, it has been shown that overexpression of the mammalian Sir2p orthologue SIRT1 down-regulates damage-response target genes, including genes that control apoptosis (Langley *et al.*, 2002; Vaziri *et al.*, 2001). Overexpression of *sir-2.1* in *C. elegans* may curtail cell damage brought on by MRC dysfunction. Sir2 family members are also linked to aging and life-span determination in nematodes; life-span increases with higher doses of *sir2.1* (Tissenbaum and Guarente, 2002). The NAD$^+$ requirement for Sir2p activity links metabolism aging; a better understanding of how these are linked may provide insight into the pathology of MRC deficiency.

In addition to redox imbalance, other pathogenic mechanisms of equal or greater importance may contribute to disease severity. Unfortunately, the roles of these other mechanisms have only been superficially addressed in this thesis. For example, we did not fully explore the importance of ROS in the pathology of the *nuo-1* mutants. The direct detection and quantification of free-radical generation and the measurement of the levels of free-radical scavenging proteins, such as superoxide dismutase (SOD) are appealing follow-up experiments. If ROS generation in MRC-deficient animals is found to overwhelm the endogenous defense mechanisms, then gene therapy approaches that lead to enhanced ROS scavenging should be considered. These may include the over-expression of the SOD genes *sod-1* and/or *sod-3*, which encode the cytosolic Cu/ZnSOD and mitochondrial MnSOD enzymes, respectively (Giglio *et al.*, 1994a; Giglio *et al.*, 1994b)

Similarly, the importance of apoptosis in pathogenesis deserves greater attention. TUNEL results reported in Chapter 2 suggest an increase in apoptotic cell death in *nuo-1* mutant animals that may be responsible for the vacuolar structures observed in the muscle tissue. The apoptotic pathway in *C. elegans* has been extensively studied and many pro- and anti-apoptotic factors are known (Putcha and Johnson, 2004). Experiments monitoring the effects of suppressing pro-apoptotic genes such as *ced-3* or *ced-4* by RNAi, or alternatively, by overexpressing anti-apoptotic genes such as *egl-1* or *ced-9* may be informative. It should be possible to determine if the tissue degeneration observed in *nuo-1* mutants is an apoptotic or a necrotic event.

Our studies with the modeled human complex I mutations have yielded other novel insights into the pathogenic mechanisms of MRC dysfunction. For example, the data in Chapter 2 suggest a direct correlation between oxidative stress and redox imbalance; pharmacological supplements that alleviate lactic acidosis also decrease sensitivity to hyperoxia and to paraquat. We have speculated that riboflavin has two modes of action: it activates the PDHC, diverting pyruvate away from lactate synthesis and stabilizes complex I, thus reducing ROS production. In contrast, the dual effects of sodium dichloroacetate are more difficult to explain. Its only known role is as a PDHC activator via inhibition of the PDH kinase.

One of the most interesting insights into complex I dysfunction is the connection discovered between complexes I and IV. As described in Chapters 2 and 3, *nuo-1* mutations not only affect the catalytic activity and assembly of complex I but also that of complex IV. The assembly and catalytic activities of both complexes are responsive to riboflavin supplementation and independent of the redox imbalance. However, the simple question remains: Why do mutations in complex I have an effect on complex IV?

It was suggested previously in the thesis that complex I deficiency may somehow affect the expression of mtDNA-encoded MRC subunits, three of which belong to complex IV. Testing this hypothesis would be difficult since the expression of mtDNA-encoded subunits can be affected at transcriptional, translational and post-translational steps of synthesis. The import machinery and the MRC are also connected by a requirement for a membrane potential for protein translocation across the mitochondrial inner membrane; $\Delta\Psi$ is derived directly from the proton-pumping action of the MRC. The majority of the mitochondrial transcription and translation machinery is nuclear-encoded and synthesized in the cytosol and relies on the mitochondrial preprotein translocases for its import and sorting to the matrix space. The preprotein import and export machineries also rely on themselves for their proper translocation and assembly into their respective mitochondrial membranes. It would be interesting to further explore the connection between MRC dysfunction and mitochondrial biogenesis. *C. elegans* has recently been used as a model system for studying mitochondrial protein translocation (Curran *et al.*, 2004). It has an evolutionarily conserved mitochondrial import machinery that includes an orthologue to the human DPP1/TIMM8a protein, which when mutated results in Mohr-Tranebjaerg syndrome or deafness/dystonia syndrome (Roesch *et al.*, 2002). Furthermore, this protein facilitates the import of mitochondrial inner membrane carriers responsible for shuttling reducing equivalents from the cytosol to the mitochondrion, such as the malate/aspartate shuttle, (Roesch *et al.*, 2004). This link between the mitochondrial import machinery and the redox balance of the cell makes a further examination of this relationship even more intriguing. It would be interesting to establish whether mitochondrial protein translocation is indeed affected by MRC dysfunction.

A global impairment of mitochondrial transcription and translation would have consequences for all MRC complexes containing mtDNA-encoded subunits, a situation that is not supported by our data. Our data suggest a complex I-mediated mechanism specific for complex IV dysfunction. This may include altered expression of nuclear-encoded genes. Complex IV catalytic activity and assembly are dependent on a number of nuclear-encoded assembly factors required for the proper insertion of its co-factors. Defects in any one of these factors have pathogenic consequences in humans. Alternatively, copper-carrying complex IV assembly factors may be more susceptible to ROS-mediated damage due to the highly-reducing environment of the matrix space exacerbated by complex I dysfunction; copper centres carried by these proteins provide potential sites for ROS generation. It would be intriguing to explore whether a complex I deficiency affects the expression or function of these assembly factors.

Continued analysis of the complex I-IV relationship in *C. elegans* may also include examination of supercomplex formation. Respiratory chain supercomplexes have been detected in a variety of organisms including mammals, yeast, plants and bacteria, suggesting similar structures may exist in *C. elegans* (Dudkina *et al.*, 2005; Eubel *et al.*, 2004; Schägger, 2002). Although there is little evidence supporting a significant physical interaction between complexes I and IV, it would be interesting to elucidate the physical organization of the nematode MRC, since there is potential for other MRC complex interactions. If a similar physical organization of the human MRC is present in worms, then the nematode model system could be used to study the effects of MRC dysfunction due to pathogenic mutations on supercomplex formation.

The studies in this thesis are a testament to the potential of the nematode model system for the study of mitochondrial disorders. However, as in any model system, there are shortcomings that should be noted for future investigations. Continued examination of MRC complex assembly has been hampered by the lack of reliable polyclonal and monoclonal antibodies that cross-react with *C. elegans* MRC proteins. The generation of a NUO-1-specific polyclonal antibody was attempted during the course of this thesis study but the antiserum was not of high enough quality for dependable identification of the protein. Furthermore, no other antibodies directed against *C. elegans* MRC proteins are available. As a result, all of the antibodies used in these investigations are directed against proteins from other species. The majority of commercially available MRC-specific antibodies are monoclonal and directed against bovine or human subunits. These antibodies are of high quality but few specifically cross-react with their *C. elegans* homologs. Interspecies antisera often require thorough testing for specificity, a time-consuming endeavor. Fortunately, some biotechnology companies are beginning to recognize *C. elegans* as a popular model system for mitochondrial study and have taken the step to include information on the cross-reactivity of their antisera against nematode proteins. The few commercially available monoclonal antibodies that do cross-react with

C. elegans MRC proteins work well but are limited in their application. For example, these antibodies are not favoured when performing blue-native gel electrophoresis, an essential method for examination of MRC complex assembly. A protein folded in its native form is less likely to expose the single epitope required for a monoclonal antibody to bind than compared to a denatured protein.

Many commercially available vital stains that detect a variety of processes related to MRC function such as apoptosis, free radicals or mitochondrial membrane potential are sometimes difficult to use in *C. elegans*. The best results are usually seen in unicellular systems that can be observed flat for optimal microscopic imaging. Furthermore, favourable specimens are relatively permeable making them amenable to post-stain washing to reduces background. In contrast, *C. elegans* is a three-dimensional, multicellular organism with a highly impermeable cuticle, making it difficult to observe in two dimensions and susceptible to high background staining. High background and non-specific staining are frequently due to contamination of the nematode with its bacterial food. This usually occurs internally by bacteria in the digestive system taking up many of the commercially available stains. Over time, the development of higher quality stains, combined with refined staining protocols specific to *C. elegans* will undoubtedly improve the efficiency and specificity of staining in the nematode.

5.2.2 Metabolic Profiling in the Study of Mitochondrial Diseases

Great progress has been made in our understanding of the molecular basis of mitochondrial disorders but their complex and multisystemic nature still makes clinical diagnosis and treatment difficult. Genetic screening can ascribe the type and location of a mutation resulting in a MRC defect but screening often suffers from the poor correlation between genotype and phenotype. Furthermore, a complete diagnosis is often challenging and requires a battery of clinical and biochemical data to supplement the genetic information. There is a need for more advanced diagnostic methods to further understand the mechanisms of mitochondrial disorders and enhance their clinical evaluation. One of these advances is the use of phosphorous (^{31}P) and proton (^{1}H) nuclear magnetic resonance (NMR) as a diagnostic tool for mitochondrial disease patients (Kuwabara *et al.*, 1994; Taylor and Radda, 1994; Rubio-Gozalbo *et al.*, 1999; Bianchi *et al.*, 2003). NMR is a quick, accurate and non-invasive method of examining the relative and absolute concentrations of metabolites and other small molecules in tissue or biofluid, creating a metabolic profile. MRC dysfunction often causes anomalous metabolism, which is the basis for many of the symptoms observed in patients with mitochondrial disorders. Changes to the metabolic profile of a patient are easily detected through NMR analysis but may go undetected by standard diagnostic methods. Acquiring metabolic profiles from patients could help identify those pathways with the greatest promise for intervention. Furthermore, NMR-based analysis could be used to gauge changes in the metabolic profiles of patients undergoing specific treatment strategies, allowing for their effectiveness or potential dangers to be assessed.

The literature contains numerous examples of the use of ^{31}P- and ^{1}H-NMR to provide additional information beyond that gained by conventional diagnostic methods for the treatment of patients with various mitochondrial and metabolic disorders. However, in some of these studies no significant correlation was found between the metabolic anomalies detected and the clinical phenotype observed. This is in part attributable to the rare and sporadic nature of these disorders; there is simply not a large enough collection of metabolic profiles from patients amassed to generate a useful database for detailed diagnosis and precise treatment. Metabolic profiling of model organisms could initiate the development of rudimentary metabolomic databases used to assist in the treatment of human metabolic disorders. In response to the increasing interest in metabolic profiling and 'metabolomics' – the study of a complete set of metabolites and intermediates within in an organism, a growing, joint clinical and laboratory framework is providing convenient access to NMR facilities (Griffin, 2004).

In this thesis we examined treatment possibilities with pharmacological supplementation and gene therapy designed to correct, bypass or compensate for specific metabolic pathways in our *nuo-1* mutants. Within these investigations, we examined the concentrations and molar ratios of a number of metabolites, such lactate, pyruvate and ATP to assess the effectiveness of our therapeutic strategies. Unfortunately, these metabolites provide only a limited evaluation of treatment effectiveness. For example, other metabolic processes might remain uncorrected or a treatment strategy, although appearing beneficial, may cause deleterious side-effects that are undetectable by conventional diagnostic assays. For these reasons, metabolic profiling of disease models would be the ideal tool to enhance these studies. Furthermore, *C. elegans* with its mammalian-like MRC and metabolism would be an excellent model system for metabolic profile experimentation.

The use of *C. elegans* in ^{31}P-NMR analyses goes back several years. These early experiments examined *in vivo* pH shifting and the relative concentrations of ATP, ADP, AMP, P_i, sugar phosphates and other phosphate-containing metabolites during nematode development (Wadsworth and Riddle, 1988; Wadsworth and Riddle, 1989). These investigations demonstrated the ease and versatility of the nematode for NMR-based studies. NMR analysis can be performed using either protein-free extract from whole-animal lysate or live intact nematodes. For this reason, the *nuo-1* mutants would be an excellent system in which to investigate the effects of pathogenic mutations and their treatment on the nematode metabolome. Fortunately, collaborations with other laboratories that specialize in NMR spectroscopy were put into practice. I was able to conduct preliminary trials of ^{31}P- and ^{1}H-NMR analysis using wild-type *C. elegans* and the three *nuo-1* mutants. These initial results confirmed what our conventional biochemical analyses revealed; all three *nuo-1* mutants had significantly elevated lactate concentrations, moderately reduced pyruvate concentrations and significant reductions in ATP content compared to wild-type *C. elegans*. In addition, the analyses revealed significant changes to metabolites that are either difficult to detect or undetectable by standard biochemical approaches. These included significant increases in glucose concentrations and an unknown phosphorous species, elevations in specific amino-acids such as alanine, serine and glycine and severe reductions in sugar phosphates in the *nuo-1* mutants in comparison to wild-type. Unfortunately, due to the inconsistent accessibility of the NMR facilities, these experiments were not repeated. As a result, further examination of mutant nematodes undergoing genetic or pharmacological therapies could not be performed during the course of my studies.

Despite the small data sets and incomplete analysis, useful insight can be garnered from these preliminary studies. As expected, major differences between the metabolic profiles of mutant and wild-type nematodes were observed and these differences extended beyond the small number of metabolites examined in the thesis work.

Furthermore, the NMR results accurately reflect the aberrant metabolism occurring in the *nuo-1* mutants since metabolic profiles determined by conventional biochemical analyses are in agreement with the NMR spectral data thus validating their use in such experiments. The data also indicate that many more metabolic defects contribute to the pathogenic phenotypes of the *nuo-1* mutants, reflecting the true complexity of pathogenesis due to MRC deficiency. Given the ease in which nematodes can be cultivated, the ever-increasing sensitivity and efficiency of NMR spectroscopy and the growing availability of powerful software to analyze, process and identify hundreds of known metabolites within a single spectral reading, continuation of this investigation would have easily generated accurate and comprehensive metabolic profiles of wild-type and complex I-deficient *C. elegans*. Generation and analysis of these profiles would undoubtedly contribute to greater understanding of the downstream pathogenic mechanisms caused by the *nuo-1* mutations.

The potential applications for these kinds of studies are endless. Any number of human MRC mutations that cause disease could be modeled in *C. elegans* and analyzed by NMR spectroscopy, quickly generating metabolic profiles that can be compared with other profiles. Common pathogenic mechanisms could easily be identified or subtle differences accurately detected, eventually leading to therapeutic approaches specific to each metabolic defect. This work would naturally progress to rational therapy development in human patients.

5.2.3 Molecular Therapy of Mitochondrial Diseases

C. elegans can not only be utilized for the development of diagnostic tools for mitochondrial disorders but can also play a significant role in the development of new molecular medicines for their treatment. Chapter 4 successfully demonstrates the potential of the nematode in the development and characterization of genetic approaches to treat MRC-associated mitochondrial disease. As a reminder, the *S. cerevisiae* cytochrome *b2* enzyme was used to help reduce the over-abundance of NADH and lactate in the nematode system while donating electrons to the MRC. The beneficial results from this study strongly support further testing of respiratory chain enzymes not normally found in the mammalian or *C. elegans* mitochondrion that can maintain the proton motive force and/or correct redox imbalance caused by MRC dysfunction.

The successful application of yeast respiratory enzymes to correct complex I dysfunction has also been demonstrated in various mammalian models with the rotenone-insensitive NADH dehydrogenase Ndi1p (Bai *et al.*, 2001; Seo *et al.*, 1998; Seo *et al.*, 2004). This enzyme serves as the single-subunit alternative to mammalian-like complex I. Further examination of the *S. cerevisiae* MRC reveals the presence of two more single-subunit mitochondrial inner membrane-bound NADH dehydrogenases Nde1p and Nde2p collectively referred to as external NADH dehydrogenases; they do not pump protons but do transfer electrons to ubiquinone (Bakker *et al.*, 2001). External NADH dehydrogenases are not found in mammalian or *C. elegans* mitochondria, but are present in many plants and fungi (Rasmusson *et al.*, 2004). While Ndi1p, an internal NADH dehydrogenase oxidizes NADH generated within the mitochondrial matrix, external NADH dehydrogenases are responsible for oxidation of cytosolic NADH. Although we suspect that the elevated NADH/NAD$^+$ ratio present in complex I-deficient *C. elegans* primarily affects the mitochondrial pool of these metabolites, the redox imbalance may extend to the cytosolic pools as well. Thus, introduction of external NADH dehydrogenases into *C. elegans*, perhaps in conjunction with cytochrome *b2* or Ndi1p expression, may further enhance NADH oxidation in a *nuo-1* mutant background while also contributing to electron flow through the MRC. A combination of internal and external NADH dehydrogenase expression in *C. elegans*, with eventual testing in mammalian cells, may prove to be an efficient and effective strategy to directly address the redox imbalance caused by complex I dysfunction.

Another oxidative enzyme common to higher plant and fungal mitochondria but not present in *C. elegans* or mammals is alternative oxidase. Alternative oxidase is a nuclear-encoded, cyanide-insensitive terminal oxidase that branches from the main MRC and directly transfers electrons from ubiquinone to molecular oxygen; it does not contribute to the proton motive force (Juszczuk and Rychter, 2003). The enzyme exists as a simple homodimeric integral inner membrane protein containing a non-heme di-iron redox center. It is usually induced in response to environmental stress factors or as a result of inhibition of the MRC *in vivo* or *in vitro* (Vanlerberghe and McIntosh, 1997). A gene therapy strategy involving expression of alternative oxidase in MRC-deficient *C. elegans* could be attempted to examine its potential as gene therapy. The role of the enzyme as a terminal oxidase would make it an ideal candidate to address complex IV deficiencies. It could also be utilized to bypass complex II and III dysfunction since alternative oxidase oxidizes ubiquinone directly, thus maintaining electron flow through the MRC. The major caveat to this strategy would be that the proton pumping functions of complexes III or IV could not be replaced by alternative oxidase. However, the enzyme may also provide protection against ROS over-production in the face of MRC dysfunction. Over-expression of alternative oxidase can decrease ROS formation in mitochondria, possibly by reducing the levels of ubiquinol and minimizing the life-time of semiubiquinone radicals, which contribute to ROS generation (Maxwell *et al.*, 1999). Use of this enzyme as a molecular therapeutic for the treatment of MRC dysfunction could minimize pathogenesis by ROS, in addition to correcting defective electron transport. The intriguing properties of alternative oxidase make the enzyme an excellent candidate for a molecular therapeutic and the *C. elegans* disease model as the ideal system in which to test it.

In addition to using interspecies respiratory enzymes to correct MRC dysfunction, one can create and test molecular therapeutic strategies by altering the expression levels of proteins native to *C. elegans*. For example, whereas pharmacological supplementation with sodium dichloroacetate benefits our *nuo-1* mutant nematodes by temporarily stimulating PDHC activity through inhibition of the PDH kinase, a genetic approach to stimulate the PDHC may prove more effective and longer lasting. This could be done in two ways: either by knocking down expression of the PDH kinase gene by RNAi or by transgenic overexpression of the PDHC through *C. elegans* transformation. The latter may prove more complicated since the PDHC in *C. elegans* is not yet characterized. Another strategy might include addressing redox imbalance by over-expression of one or more components of the glycerol-phosphate shuttle. The mitochondrial element of this mechanism is represented by the FAD-dependent glycerol-3-phosphate (G-3-P) dehydrogenase, which catalyzes the oxidation of G-3-P to dihydroxyacetone-phosphate (DHAP) while transferring electrons to ubiquinone. The enzyme is located on the IMS face of the mitochondrial inner membrane. The action of the mitochondrial G-3-P dehydrogenase is coupled to the activity of the cytosolic NAD^+-dependent G-3-P dehydrogenase, which catalyzes the conversion of DHAP back to G-3-P while oxidizing cytosolic NADH to NAD^+. Over-expression of one or both of these glycerol phosphate shuttle constituents may reduce the amount of cellular NADH in a complex I-deficient background. Testing of this therapeutic mechanism would be easy enough in the *C. elegans* system with the caveat that these enzymes have yet to be characterized in the nematode.

Continued work with genetic approaches to address MRC dysfunction in *C. elegans* may eventually branch into the exploration of treating mitochondrial disorders due to mtDNA mutation. Pathogenesis of these kinds of disorders is heavily influenced by the mutational load and heteroplasmy of mtDNA defects. Gene therapy strategies for their treatment would therefore focus on either reducing the mutational load of mtDNA or possibly correcting the mutation itself within the mitochondrion; these mechanisms are more complex than the relatively simple correction of nuclear defects. The genetic amenability of *C. elegans* may provide the means to perfect the methods needed for stable and heritable transfection of mammalian-like mitochondria with DNA, a feat already accomplished in yeast (Butow *et al.*, 1996) but yet to be consistently demonstrated in mammalian mitochondria. As more of the *C. elegans* proteome is characterized, especially those proteins involved in mitochondrial functions, the potential of the nematode awaits those needing a transitional model system to test new therapeutic strategies before advancing to mammalian tissue systems and to clinical application.

5.3 Concluding Remarks

The study of mitochondrial diseases can be complicated and enigmatic at times. The nature of these disorders requires an experimental system that has the potential to address the molecular complexities of their pathogenesis while being simple enough to provide information quickly and efficiently. I feel the work in this thesis has demonstrated the true potential of *C. elegans* as a model system to investigate not only the molecular details of MRC dysfunction in a whole multicellular organism but also its diagnosis and treatment. The relevance of this research to future medical application is undeniable. We have demonstrated that pathogenic human mutations can be successfully modeled in *C. elegans* and that their effects appear to accurately reflect the molecular mechanisms of human disease. Furthermore, we observed aspects of pathogenesis that have not been and most likely could not have been identified in human patients due to clinical limitations. Finally, we have demonstrated that *C. elegans* can be used as a valuable and effective system for the development of novel molecular therapies that may hold promise for safe and effective treatment of mitochondrial disorders in the future.

5.4 Bibliography

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